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Characterization of the Epsin Homolog EpnA in *Dictyostelium discoideum*

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Characterization of the Epsin Homolog EpnA in *Dictyostelium discoideum*

by

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Dedication

To Parrish

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Characterization of the Epsin Homolog EpnA
in *Dictyostelium discoideum*

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Clathrin-coated pits on the plasma membrane invaginate into coated vesicles to internalize receptors and membrane. The clathrin adaptor epsin contains an amino-terminal ENTH domain that binds $PI(4,5)P_2$ and a carboxy-terminal domain that binds clathrin, and accessory proteins such as AP2. Here, we assessed how inter- and intramolecular factors affect the contribution of epsin to coated-pit function in living cells. We found *Dictyostelium* epsin was not required for global clathrin function, but plays an essential role in spore development. We demonstrated that clathrin, but not AP2, was critical for epsin to associate with clathrin-coated pits. We found that the carboxy-terminal region of epsin was essential, but not sufficient, for targeting epsin within clathrin-coated pits on the plasma membrane. In addition to targeting epsin to the membrane, the amino-terminal ENTH domain regulates the interaction between epsin and clathrin, an essential property that cannot be replaced by an alternate

$PI(4,5)P_2$ binding domain. Moreover, the ENTH domain facilitates the functional interaction between clathrin and actin during late stages of endocytosis, possibly by regulating the activity of the adaptor Hip1r. Both the ability to bind $PI(4,5)P_2$ and another function mediated by residue T107 are critical for the activity of the ENTH domain. Our results support a model where the ENTH domain coordinates with the clathrin-binding C-terminal domain to allow a dynamic interaction of epsin with coated pits. Furthermore, we propose that the ENTH domain of epsin facilitates the membrane recruitment and phosphorylation of Hip1r, which in turn mediates the productive interaction of clathrin with the actin cytoskeleton at the plasma membrane.

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Chapter 1: General Introduction

1.1 Endocytosis in eukaryotic cells

Biological membranes are a foundational component of all cells. The outer plasma membrane provides critical separation between the intracellular and extracellular environment. The intracellular environment is carefully controlled by limiting what passes through the plasma membrane. Many small molecules, such as sugars, ions, and amino acids are transported across the plasma membrane by specific channels and pumps. Other substances are brought into the cell via endocytosis, where areas of the membrane invaginate and pinch off to form membrane-bounded compartments called endosomes (Lodish, 2003). In addition to internalizing extracellular cargo, endocytosis changes the protein and lipid composition of the plasma membrane, and is therefore involved in a myriad of cellular processes, such as immune surveillance, uptake of nutrients, and processing of extracellular signals.

1.2 Clathrin-mediated endocytosis

Specific transmembrane and extracellular proteins are internalized by clathrin-mediated endocytosis. In clathrin-mediated endocytosis, clathrin forms a coated pit on the plasma membrane around this endocytic cargo in association with several accessory factors. These coated pits bud off into the cell, creating small clathrin-coated vesicles. Clathrin-mediated endocytosis is important for internalizing signaling receptors such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) (Miljan and Bremer, 2002). Clathrin-mediated endocytosis is used to process extracellular ligands, allowing the cell to absorb

relatively high amounts of solutes present in low concentrations in the extracellular environment. In addition, clathrin-mediated endocytosis retrieves plasma membrane components for recycling and/or degradation and is essential for synaptic vesicle recycling in neurons (Brodin *et al*., 2000).

Because clathrin-mediated endocytosis is a ubiquitous and essential process in all eukaryotes, many pathogens take advantage of this pathway to gain entry into the cell. One example is the human adenovirus, which binds to membrane receptors in lung tissue and triggers endocytosis of the pathogen along with the receptor (Meier and Greber, 2004). Moreover, small defects and mutations in this process can lead to serious medical conditions. Familial hypercholesterolemia, congenital hyperinsulinism, and neonatal diabetes mellitus are examples of diseases caused by genetic mutations that disrupt the clathrin-dependent internalization of critical membrane receptors (Norman *et al*., 1999; Partridge *et al*., 2001; Sivaprasadarao *et al*., 2007). Certain clathrin-associated proteins are also implicated in specific diseases. For example, epsin is linked to schizophrenia (Liou *et al*., 2006; Tang *et al*., 2006), Hip1/Hip1r may be involved in Huntington's disease (Kalchman *et al*., 1997), and CALM was identified as a lymphoid myeloid leukemia gene (Dreyling *et al*., 1996).

Although many of the components of clathrin-mediated endocytosis have been identified and extensively studied, the macromolecular mechanisms for how and where clathrin and its associated proteins work together to form functional coated pits on the plasma are not well understood.

1.2.1 Clathrin

Clathrin coats are composed of many individual clathrin triskelia, or three-legged structures, each containing three heavy chains and three light chains. Each

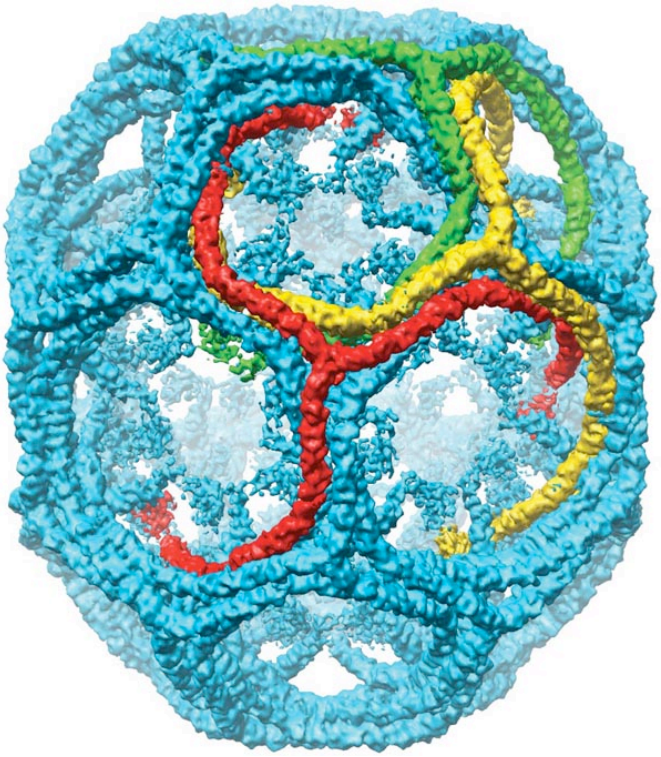

Figure 1.1 Assembled clathrin cage as determined by electron cryomicroscopy.

heavy chain consists of a carboxy-terminal hub domain, a series of helices forming a "leg", and an amino-terminal globular domain (Ungewickell, 1999). The hub domains of the three heavy chains interact to form the stable center of the triskelion, while the "legs" interact with other triskelia to form a lattice-like structure on the plasma membrane (Fotin *et al*., 2004b). The amino-terminal domain forms a seven-bladed β-propeller structure that interacts with other components of the coated pit (ter Haar et al., 2000; ter Haar et al., 1998).

Clathrin light chain is much smaller than the heavy chain (25kDa vs. 192kDa), but is important for clathrin function. Clathrin light chain can stabilize assembled heavy chain triskelions (Ybe *et al.*, 2007b). Mutation or deletion of the light chain compromises clathrin function and causes phenotypic deficiencies in yeast and *Dictyostelium* (Huang et al., 1997; Wang et al., 2003; Wang et al., 2006b).

Careful analysis has shown that clathrin light chain plays an important regulatory function. An acidic motif at the amino-terminus of clathrin light chain binds to the carboxy-terminal hub domain of clathrin heavy chain (Liu *et al.*, 1995; Ybe *et al.*, 1998). Clathrin heavy chains alone can self-assemble into lattices *in vitro* at physiological pH (Liu *et al.*, 1995), but addition of clathrin light chain inhibits this self assembly by preventing the formation of high-affinity salt bridges between heavy chains. (Ybe *et al.*, 1998) This allows the formation of clathrin coats to be precisely regulated in the cell.

In addition to regulating clathrin assembly, clathrin light chain also mediates the interaction of clathrin with several important accessory factors. Hcs70, a protein important for uncoating of clathrin vesicles after internalization, binds to a central helical region of clathrin light chain. (Brodsky *et al.*, 1991) The actin-associated adaptors Hip1/Hip1r also bind directly to clathrin light chain (Legendre-Guillemin *et al.*, 2002). Thus the heavy and light chains of clathrin work together to assemble and organize into functional coated pits on the membrane.

1.2.2 Life cycle of a clathrin-coated pit

Clathrin assembly into coated pits is a dynamic process. Constant free exchange of clathrin triskelia and other accessory factors remodels the growing pit as it assembles at plasma membrane with other accessory factors, forms a coat around

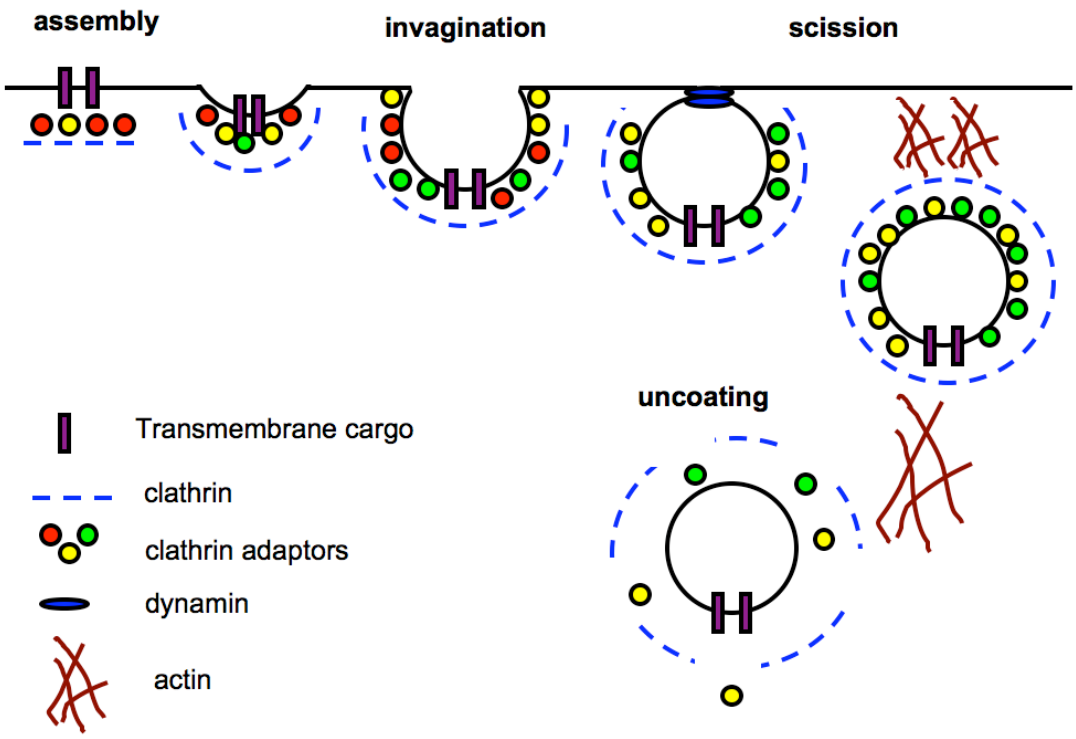

Figure 1.2 Schematic representation of clathrin-mediated endocytosis.

Clathrin and adaptors assemble around transmembrane cargo. The coated pit invaginates and eventually "pinches off" into the cytosol. The vesicle is then uncoated before delivery to intracellular targets.

the endocytic cargo, invaginates, and pinches off from the membrane. The life cycle of a coated pit can be roughly divided into 4 stages: assembly, invagination, scission, and uncoating.

1.2.2.1 Assembly

The first stage of clathrin-mediated endocytosis involves assembling clathrin into lattices at the endocytic site. Although clathrin can self-assemble into lattices *in vitro*, several additional proteins are required to correctly recruit clathrin to endocytic targets at the plasma membrane *in vivo* (Brodsky et al., 2001; Liu et al., 1995). These additional proteins are called clathrin accessory proteins. If these accessory proteins also aid in the assembly of clathrin lattices at the membrane, they are referred to as "assembly proteins." The principle assembly protein is AP2. Assembly proteins frequently bind to endocytic cargo as well as to clathrin. During the assembly phase of clathrin vesicle formation, various accessory proteins and recruit and organize clathrin around clusters of transmembrane cargo. Clathrin accessory proteins generally have many binding motifs and domains to link clathrin with endocytic cargo, regulate vesicle size, and tether the growing pit to the membrane. For this reason they are also referred to as "clathrin adaptors," meaning they link clathrin to other proteins or cellular structures (Owen *et al*., 2004).

The process of clathrin pit assembly is not linear; rather several components are independently targeted to the same endocytic site, then interact with each other to organize around the cargo and recruit additional factors. On the plasma membrane, the phospholipid phosphatidylinositol (4,5) *bis*-phosphate ($PI(4,5)P_2$) is enriched at endocytic sites and plays a key role in endocytosis (Jost *et al*., 1998). Most of the

major clathrin adaptors that function at the plasma membrane contain domains for binding to $PI(4,5)P_2$ (Krauss and Haucke, 2007).

In addition, the cytoplasmic tails of transmembrane receptors may contain internalization signals recognized by components of the endocytic machinery. Prominent among these motifs are [D/E]XXL[L/I], YXXΦ, and FXNPXY (Matter *et al.*, 1994; Ohno *et al.*, 1995; Ungewickell and Hinrichsen, 2007). Alternatively, cargo may be tagged for internalization by mono- or poly-ubiquitination (Traub and Lukacs, 2007). Adaptor proteins such as AP2 and epsin recognize these signals and promote clathrin assembly around these receptors (Hawryluk et al., 2006; Ohno et al., 1995).

1.2.2.2 Invagination

Invaginating a coated pit prior to vesicle scission requires a high degree of membrane deformation (McMahon and Gallop, 2005). No one protein or lipid is solely responsible for bending the membrane, but rather clathrin and adaptors work synergistically to induce and maintain membrane curvature (Ungewickell and Hinrichsen, 2007). The ENTH domain, which binds to $PI(4,5)P_2$, actively induces positive membrane curvature by inserting an amphipathic helix into the inner leaflet of the plasma membrane (Ford *et al.*, 2002; Stahelin *et al.*, 2003). Clathrin itself can assemble into different lattice conformations depending on the degree of curvature (Brodsky *et al.*, 2001; Nossal, 2001). Other domains, such as the BAR domain of amphiphysin, preferentially bind to curved membranes (Peter *et al.*, 2004). The lipid composition of the membrane can also be modified to favor positive or negative curvature (Brown *et al.*, 2003; Kooijman *et al.*, 2005). All of these factors work together to create a coated pit that is sufficiently invaginated to form a coated vesicle.

1.2.2.3 Scission

Once a coated pit has become deeply invaginated, the "neck" of the pit must be constricted so that the vesicle can pinch off. The GTPase dynamin is the "pinchase" that provides the mechanical force necessary to complete vesicle scission (Sweitzer and Hinshaw, 1998). Indeed, the well-known temperature-sensitive *shibire* mutant of *D. melanogaster* cannot complete vesicle scission (and therefore vesicle recycling) at the restrictive temperature, resulting in paralysis (Grigliatti *et al.*, 1973; Kosaka and Ikeda, 1983; van der Bliek and Meyerowitz, 1991). Electron microscopy of these mutants reveals membranes covered with late-stage, deeply invaginated coated vesicles (Koenig and Ikeda, 1989). Thus, dynamin plays an essential role in vesicle scission.

However, Arp2/3, N-WASP, endophilin, and auxilin are also recruited to the coated pit just before scission. Many of these proteins bind to actin, and there is a growing body of evidence indicating actin plays a key role during vesicle scission, perhaps by providing tensile stress that pulls the vesicle inward. This force may facilitate the contraction of dynamin around the neck of the vesicle (Roux *et al.*, 2006). Alternatively, the concentration of proteins at the vesicle neck could create a phase separation between the membrane lipids, creating a weak spot that "snaps" when tension is applied (Liu *et al.*, 2006).

1.2.2.4 Uncoating

After scission, the clathrin-coated vesicle must be uncoated before the vesicle cargo is delivered to its intracellular target. This is accomplished via the activity of Hsc70 and auxilin (Jiang *et al.*, 1997; Ma *et al.*, 2002). Hsc70 is an ATPase with many functions in the cell, but it can only function in clathrin-mediated endocytosis if

auxilin is present (Prasad *et al.*, 1993; Ungewickell *et al.*, 1995). Auxilin is a monomeric clathrin accessory protein that binds phospholipids and contains a conserved J-domain which interacts with Hsc70 (Jiang *et al.*, 2003; Lee *et al.*, 2006). Auxilin also binds directly to clathrin (Scheele *et al.*, 2001).

Detailed structural studies *in vitro* of auxilin and Hsc70 with assembled clathrin cages suggest a molecular mechanism for vesicle uncoating. Auxilin binds on the inner surface of the clathrin coat near several critical joints in the triskelia, causing measurable distortions in the clathrin coat (Fotin *et al.*, 2004a). Hsc70 binds the J-domain of auxilin near the amino-terminal "hook" of clathrin heavy chain, allowing individual clathrin triskelia to be "pried away" from the clathrin basket (Fotin *et al.*, 2004a; Heymann *et al.*, 2005).

1.3 ACTIN IN CLATHRIN-MEDIATED ENDOCYTOSIS

Actin dynamics provide the mechanical force necessary for many stages of endocytosis. Actin provides this force via two mechanisms. The first involves motor proteins, such as myosins, that attach to intracellular cargo while binding polymerized actin filaments through an amino-terminal head (Krendel and Mooseker, 2005). These motor proteins function by translating the chemical energy of ATP hydrolysis in the head domain into mechanical energy that allows them to take "steps" along an actin filament, physically pulling cargo along with it (Alberts, 2002). Various cargo binds specifically to different motor proteins (Vale, 2003), and different classes of motor proteins can move either to the barbed/plus end or pointed/minus end of an actin filament, although the majority of motors are barbed/plus end directed (O'Connell *et al.*, 2007).

Myosin VI has been specifically implicated in clathrin-mediated endocytosis (Buss *et al.*, 2001a; Spudich *et al.*, 2007). Because it is a pointed/minus end directed myosin and most cortical actin around endocytic sites is oriented with the plus end pointing towards the membrane, myosin VI is uniquely able to direct endocytic vesicles towards the interior of the cell away from the plasma membrane (Buss *et al.*, 2001b). Myosin VI colocalizes with clathrin pits on the plasma membrane and physically binds to coat components (Buss *et al.*, 2001a; Spudich *et al.*, 2007). Thus myosin VI may also aid in the invagination of clathrin pits before scission.

The other mechanism by which actin provides mechanical force during endocytosis is through direct polymerization of F-actin, which can literally push a vesicle away from the membrane (Giardini *et al.*, 2003; Upadhyaya *et al.*, 2003). Bursts of actin polymerization accompany the final stages of vesicle scission (Merrifield *et al.*, 2002). Treatment of cells with actin-depolymerizing drugs arrests clathrin pits on the membrane (Merrifield *et al.*, 2005; Newpher *et al.*, 2005). This suggests that actin is important for the final stages of invagination and scission. Both mechanisms of force generation operate during endocytosis.

A great deal of our understanding about the role of actin during clathrin-mediated endocytosis comes from extensive genetic studies in yeast. In this system, clathrin-mediated endocytosis occurs predominantly at cortical patches on the plasma membrane (Kaksonen *et al.*, 2003; Kaksonen *et al.*, 2005; Newpher *et al.*, 2005). These cortical patches have an initial stationary phase, then transition from slow movement to rapid movement prior to internalization (Kaksonen *et al.*, 2003).

Elegant live imaging of yeast cells expressing GFP- and RFP-labeled endocytic proteins has yielded a detailed temporal picture of actin assembly at endocytic sites. First, the yeast WASP homolog Las17p is recruited to clathrin pits,

followed by Sla1p, Sla2p, End3p, and Pan1p (Kaksonen *et al.*, 2003; Kaksonen *et al.*, 2005; Newpher *et al.*, 2005). Of these, two interact directly with actin. Pan1p binds to F-actin and stimulates Arp2/3-mediated actin nucleation (Duncan et al., 2001; Huang and Cai, 2007; Toshima et al., 2005). Sla2p, the yeast homolog of Hip1r, also binds F-actin and is important for a functional coupling of clathrin to the cytoskeleton, including the regulation of Pan1p function (Kaksonen *et al.*, 2003; Newpher and Lemmon, 2006; Toshima *et al.*, 2007). Following the recruitment of these early endocytic proteins, there is a rapid recruitment of proteins that directly mediate actin polymerization, including Abp1p, Arc15p, and Arp2/3 (Kaksonen *et al.*, 2003; Kaksonen *et al.*, 2005; Newpher *et al.*, 2005). This recruitment is associated with an increase in patch motility, followed by rapid internalization (Kaksonen *et al.*, 2003). Mutations in the actin-associated endocytic proteins leads to defects in actin polymerization and vesicle formation, indicating that clathrin and actin are closely linked during endocytosis in yeast (Kaksonen *et al.*, 2005).

These findings in yeast dovetail nicely with live imaging of actin dynamics during endocytosis in mammalian cells. As in yeast, clathrin-coated pits have both stationary and mobile phases, with mobility increasing just before vesicle scission (Merrifield *et al.*, 2002; Rappoport *et al.*, 2006). Just before scission, dynamin, N-WASP, and cortactin are recruited to the pit, followed by a brief burst of actin polymerization (Merrifield et al., 2002; Merrifield et al., 2005; Merrifield et al., 2004). Blocking actin polymerization causes failure in all stages of clathrin pits, from assembly to scission, as well as limit the lateral mobility of clathrin pits (Merrifield *et al.*, 2005; Yarar *et al.*, 2005). Thus actin plays an important role throughout clathrin-mediated endocytosis.

1.4 ROLE OF ADAPTORS IN CLATHRIN-COATED PITS

Each stage of clathrin-mediated endocytosis involves a host of proteins in addition to clathrin. These proteins are generally called clathrin accessory proteins, but may also be called adaptor proteins. These proteins help to organize the coated pit and link clathrin to the plasma membrane. Adaptors also assist in the selective incorporation of endocytic cargo, or the receptors and proteins to be internalized. An adaptor may be defined as a protein that links clathrin to endocytic cargo (Pearse, 1988; Robinson, 2004), links clathrin to both cargo and the membrane (Wendland, 2002), or more generally a protein that links clathrin to any lipid or protein component at the membrane (Owen *et al.*, 2004).Thus, Hip1r and amphiphysin, which do not bind endocytic cargo, may also be referred to as clathrin adaptors (Itoh and De Camilli, 2006; Szymkiewicz et al., 2004). However, most clathrin adaptors are involved in two or more of the following processes in addition to binding clathrin: Cargo selection, membrane binding, and coated pit organization. These interactions form a complex, dynamic network at the site of endocytosis.

1.4.1 Cargo selection

Although clathrin has been reported to bind directly transmembrane cargo at the plasma membrane, generally cargo is recognized by clathrin adaptors (Bonifacino and Traub, 2003; Kibbey et al., 1998). Transmembrane cargo frequently contains one of three recognition sequences on their cytoplasmic tails, but they may also be ubiquitinated as an internalization signal. We will consider each of these modes of cargo selection in turn.

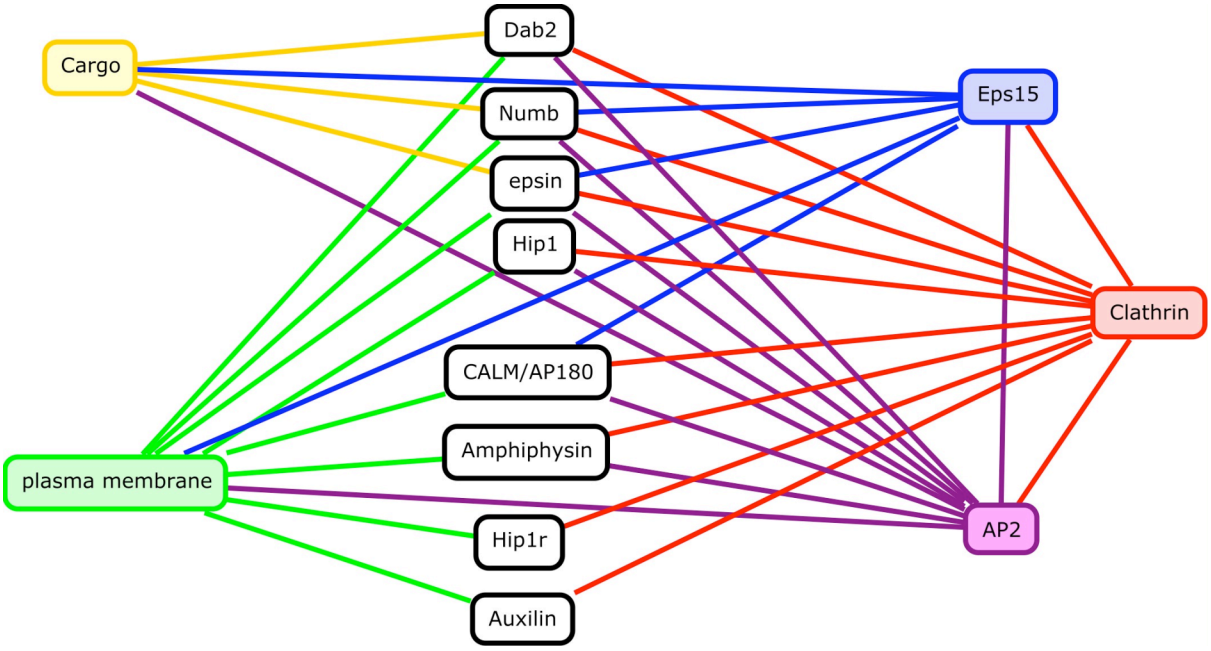

Figure 1.3 Schematic representation of the network between components of clathrin-coated pits.

Each box represents a protein, with the exception of the green "plasma membrane" box, which represents interactions with lipids of the plasma membrane. Each colored line represents a demonstrated direct interaction.

1.4.1.1 YXXΦ

The YXXΦ motif, where Φ is any hydrophobic residue, is one of the best-characterized internalization signals (Bonifacino and Traub, 2003; Robinson, 2004). Originally identified as an important signal for internalization of mannose 6-phosphate/insulin-like growth factor-II receptor (Canfield *et al.*, 1991; Jadot *et al.*, 1992), the YXXΦ motif has also been found on a wide range of transmembrane proteins (Bonifacino and Traub, 2003). In addition, YXXΦ mediates the intracellular sorting to lysosomes (Harter and Mellman, 1992; Williams and Fukuda, 1990). This

motif is recognized by the μ2 subunit of the AP2 adaptor complex on the plasma membrane (Collins *et al.*, 2002). This site is partially blocked, requiring AP2 to undergo a conformational change before it can bind to cargo with this motif. This conformational change is induced when AP2 binds to $PI(4,5)P_2$ on the plasma membrane (Honing *et al.*, 2005). Since the YXXΦ motif is found on many proteins in the cell, this regulation may help to prevent AP2 from binding to this motif inappropriately.

1.4.1.2 [F/Y]XNPXY

[F/Y]XNPXY was the first sequence-based internalization signal to be identified on the cytoplasmic tail of a transmembrane receptor (Bonifacino and Traub, 2003; Chen et al., 1990). The sequence was originally determined to be NPXY, but this was later expanded to include an aromatic residue upstream of NPXY (Collawn *et al.*, 1991; Perez *et al.*, 1999). While clathrin heavy chain has been reported to interact directly with the [F/Y]XNPXY motif (Kibbey *et al.*, 1998), most likely, adaptors are predominantly responsible for incorporating cargo with this signal into coated pits (Bonifacino and Traub, 2003). The μ2 subunit of AP2 binds to this sequence, although it binds in a different site than YXXΦ (Boll *et al.*, 2002).This motif is also recognized by the cargo-specific adaptor Dab2 (Owen *et al.*, 2004). Dab2 recognizes the [F/Y]XNPXY motif via a phosphotyrosine-binding domain (PTB). While a related protein, Dab1, is specific for phosphorylated tyrosine, Dab2 appears to preferentially bind the unphosphorylated form of this motif (Mishra et al., 2002; Morris and Cooper, 2001; Oleinikov et al., 2000).

1.4.1.3 [D/E]XXL[L/I]

The [D/E/]XXl[L/I] motif, also called a dileucine motif, was discovered when a peptide segment containing no tyrosine residues could still induce internalization (Letourneur and Klausner, 1992). This motif is found on several receptors, including insulin receptor, $GABA_A$ ligand-gated chloride channel, and the T-cell antigen receptor (Hamer *et al.*, 1997; Herring *et al.*, 2003; Szymczak and Vignali, 2005). This motif is also important of the internalization and sorting of the HIV-1 Nef (Bresnahan *et al.*, 1998; Craig *et al.*, 1998; Greenberg *et al.*, 1998).

While it is generally agreed that AP2 recognizes the [D/E/]XXl[L/I] motif at the plasma membrane, there is some debate about exactly how and where this motif binds on the AP2 complex. The HIV-1 Nef protein binds to the μ2 subunit (Craig *et al.*, 2000), while GLUT8, a glucose transporter, has been show to bind to the β2 subunit (Schmidt *et al.*, 2006). Perhaps most surprising is the recent finding that various dileucine motifs are best recognized by α2 and σ2 hemicomplex (Doray *et al.*, 2007).

1.4.1.4 Ubiquitination

Mono- and polyubiquitination is a prominent internalization signal, especially in yeast (Bonifacino and Traub, 2003; Bonifacino and Weissman, 1998; Rotin et al., 2000). The internalization of mammalian receptors, such as EGFR and the epithelium sodium channel, are also linked to ubiquitination (Levkowitz *et al.*, 1999) (Rotin, 2000). The ability of epsin to recognize ubiquitinated cargo will be discussed later in the chapter.

1.4.2 Plasma membrane-binding domains

The membranes of different cellular compartments are enriched for certain characteristic lipids. Membrane binding domains coordinate assembly and organization at specific locations in the cell through their specificity to particular lipids. Domains specific for $PI(4,5)P_2$ are targeted to the plasma membrane. Membrane-binding domains can sense and induce membrane curvature as well as facilitate conformational changes of adaptor proteins. Three prominent membrane interaction domains found in clathrin adaptors are the BAR domain, the ENTH domain, and the ANTH domain.

1.4.2.1 BAR domain

The BAR (*B*in, *a*mphiphysin, *R*vs) domain is a banana-shaped domain formed from several α-helices. BAR domains dimerize into large crescents which bind to highly-curved membranes (Peter *et al.*, 2004). While the BAR domains of amphiphysin and endophilin are the most well-characterized, a wide array of proteins contain BAR domains, not all of which are implicated in clathrin-mediated endocytosis (Itoh and De Camilli, 2006; Szymkiewicz et al., 2004).

The original BAR domain, as defined by its crystal structure, was found to bind preferentially to curved membranes, and *in vitro* studies of BAR domains revealed a potent ability to induce membrane curvature and tubulate lysosomes (Peter et al., 2004; Razzaq et al., 2001; Takei et al., 1999). This curvature-inducing activity is greatly enhanced by a short stretch of amino acids immediately N-terminal to the BAR domain (Peter *et al.*, 2004). These amino acids are induced to form an amphipathic helix when the BAR domain is bound to the membrane (Blood and Voth,

2006; Fernandes *et al.*, 2008). The entire curvature-sensing and curvature-inducing structure is called the N-BAR domain (Peter *et al.*, 2004).

The degree of curvature in the dimer structure suggests that BAR domain-containing clathrin adaptors, such as amphiphysin and endophilin, function at the late stages of endocytosis when there is deep invagination (Chen *et al.*, 2004; Peter *et al.*, 2004).

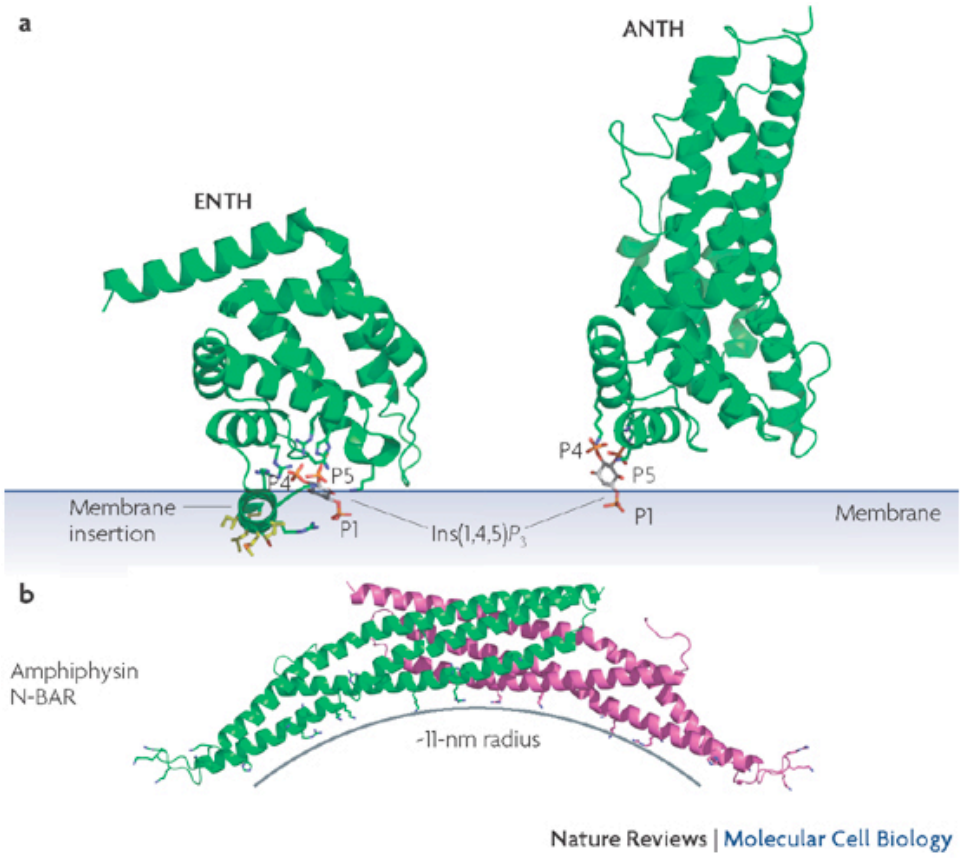

Figure 1.4 Structure of ENTH, ANTH, and BAR domains

(A) Epsin ENTH domain (PDB 1H0A) and AP180 ANTH domain (PDB 1HFA) shown in comparable orientations. (B) BAR domain from Drosophila melanogaster amphiphysin (PDB 1URU). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology (Lemmon, 2008), copyright 2008.

1.4.2.2 ENTH domain

The ENTH domain (*E*psin *N*-*T*erminal *H*omology) is a conserved region of about 140 amino acids at the amino-terminus of the epsin family of adaptors (Kay *et al.*, 1999). This domain binds specifically to PI(4,5)P_2 by forming a pocket that binds to both the headgroup and backbone of the lipid (Itoh *et al.*, 2001). The original crystal structure of the ENTH domain revealed a series of 7 α-helices (Hyman *et al.*, 2000; Koshiba *et al.*, 2002). Closer examination revealed that an eighth α-helix, called helix 0, formed when the ENTH domain bound to PI(4,5)P_2 (Ford *et al.*, 2002) (Stahelin *et al.*, 2003). This helix is amphipathic, with several exposed hydrophobic residues that insert into the membrane, causing membrane curvature. Accordingly, the ENTH tubulates liposomes *in vitro* (Ford *et al.*, 2002).

The ENTH domain may also be found on clathrin adaptors at the trans-Golgi network, but these ENTH domains have a different lipid specificity from those that function at the plasma membrane (Chidambaram et al., 2004; Kalthoff et al., 2002b).

1.4.2.3 ANTH domain

The ANTH domain (*A*P180 *N*-*T*erminal *H*omology) is found on AP180, CALM, HIP1/R families of adaptors. While related to the ENTH domain, the ANTH domain is structurally distinct (Ford *et al.*, 2002; Stahelin *et al.*, 2003). The ANTH domain is composed of a series of α-helices and binds to phosphatidylinositides in the membrane (Ford *et al.*, 2001; Stahelin *et al.*, 2003). The ANTH domain of AP180 binds to the headgroup of PI(4,5)P_2 via an exposed lysine and histidine residue (Ford *et al.*, 2001). While the specificity of the ANTH is usually for PI(4,5)P_2, the ANTH

domain of Hip1r has been shown to bind $PI(3,4)P_2$ and $PI(3,5)P_2$ (Hyun *et al.*, 2004). However, the ANTH domain does not undergo any significant conformational change when bound to lipids, nor does the ANTH domain sense or induce membrane curvature (Ford *et al.*, 2002; Stahelin *et al.*, 2003).

1.4.2.4 Other membrane interaction mechanisms

Clathrin adaptors may also interact with the membrane through other domains and motifs. In β-arrestin, a series of β-sheets forms a basic groove that interacts with phosphoinositides (Gaidarov *et al.*, 1999), while auxilin interacts with phospholipids via a PTEN-like domain (Lee *et al.*, 2006).

These membrane interactions can function in both recruitment to the membrane and regulation of endocytic events. For example, AP2 interacts with $PI(4,5)P_2$ through two different binding sites: one on the truck of the α subunit, and the other on the medium μ2 subunit (Collins *et al.*, 2002). The μ2 subunit also binds to endocytic cargo. However, the cargo binding site is masked and is not exposed until a conformational change induced by $PI(4,5)P_2$ occurs (Collins *et al.*, 2002; Honing *et al.*, 2005). Thus cargo recognition by AP2 can be restricted to the $PI(4,5)P_2$-rich plasma membrane, preventing inappropriate recruitment at other intracellular sites.

1.4.3 Clathrin assembly and organization

A common feature of clathrin adaptors is their ability to network with each other via short binding motifs. These motifs are specific, but bind with low affinity. Many binding events must occur have a cumulative effect on maintaining protein-

protein interactions (Owen *et al.*, 2004). This system allows for dynamic reorganization as the coated pit matures and invaginates. These motifs also promote assembly of clathrin at the membrane and may mediate the temporospatial recruitment and organization of clathrin and adaptors in a maturing coated pit.

The most prevalent of these motifs are discussed below.

1.4.3.1 Clathrin-binding motifs

The ability to bind clathrin is a defining feature of clathrin adaptors. The first clathrin-specific binding motif identified was the type I clathrin box, defined as LΦXΦ*, where Φ is a bulky hydrophobic residue and * is an acidic residue (Dell'Angelica *et al.*, 1998; Owen *et al.*, 2004). This motif is present is a wide range of proteins that interact with clathrin on the plasma membrane and at other locations in the cell (Robinson, 2004). Prominent adaptors containing this motif include AP2 (Shih *et al.*, 1995), AP180 (Ford *et al.*, 2001), epsin (Drake and Traub, 2001), and amphiphysin (Ramjaun *et al.*, 1997). The type I clathrin-binding motif interacts with the amino-terminal β-propeller domain of clathrin heavy chain by binding in the groove between two "blades" of the β-propeller structure (ter Haar *et al.*, 2000).

A second prevalent clathrin-binding motif is the [D/S]LL motif, which also binds to the amino-terminal domain of clathrin heavy chain (Morgan *et al.*, 2000). Many of the adaptors that contain a type I clathrin box also contain this motif, including AP2 and AP180 (Morgan *et al.*, 2000). A third motif, the PWDLW or W-box motif is found on amphiphysin and binds to the clathrin propeller domain at a site distinct from the type I motif (Miele *et al.*, 2004).

1.4.3.2 AP2-binding motifs

Binding motifs for AP2 are nearly as prevalent as binding motifs for clathrin. The majority of clathrin adaptors that function early in coated pit formation bind to AP2 (Traub, 2003). The best-characterized AP2-binding motif is the DPF/W motif, which binds to the "platform" subdomain in the ear appendage of the α subunit (Owen *et al.*, 1999; Traub *et al.*, 1999). While the DPF/W motif can occur alone, adaptors often carry multiple DPF/W repeats. Human epsin1, for example, contains 8 DPW repeats in its carboxy-terminal region.

The FXDXF motif, found in AP180 and synaptojanin, also binds to the platform region of the α-ear (Brett *et al.*, 2002). The WXXF motif, on the other hand, interacts with the β-sandwich subdomain of the α-ear (Ritter *et al.*, 2004). The different binding sites may assist in the regulation of AP2 binding by allowing some proteins to bind AP2 simultaneously while other proteins must compete for the same binding sites, depending on the motif each protein carries.

The ear domain of the β2-subunit also plays a role in networking with other adaptors. Many adaptors also interact specifically with the β2 ear appendage, most likely on the top and side of the β2 ear (Edeling *et al.*, 2006; Schmid *et al.*, 2006).

1.4.3.3 EH domain-binding motifs

EH domain-containing proteins are found an all stages of the endocytic process, including eps15, intersectin, and EHD1-4 (Polo *et al.*, 2003). The EH domain mediates protein-protein interactions by binding specifically to the motif NPF (Salcini *et al.*, 1997). This motif is found on the endocytic proteins epsin, AP180, and numb, among others, all of which have been shown to interact with EH domain-containing proteins (Confalonieri and Di Fiore, 2002). As with other short peptide motifs, the

EH-NPF interaction is of relatively low affinity, requiring multiple interactions to form a stable network (Paoluzi *et al.*, 1998; Yamabhai *et al.*, 1998). Many EH-containing proteins have multiple EH domains, and likewise the NPF motif is usually present in several copies, thus increasing the number of possible contacts between the proteins. Recent evidence also suggests that one EH domain can bind to two NPF motifs simultaneously with much higher affinity than a single NPF-EH domain interaction (Rumpf *et al.*, 2008).

1.5 ADAPTORS OF INTEREST TO THIS STUDY

1.5.1 AP2

AP2 is the principal clathrin adaptor at the plasma membrane. AP2 recognizes transmembrane cargo (Pearse, 1988), promotes clathrin assembly (Ahle and Ungewickell, 1989), binds to the plasma membrane (Collins *et al.*, 2002), and serves an organizational hub for many other endocytic proteins (Hinrichsen et al., 2003; Owen et al., 1999). The AP2 complex is formed from four subunits: α, β2, μ2, and σ2. Each subunit contributes to the function of the AP2 complex, and each will be considered in turn.

1.5.1.1 α subunit

The α-adaptin subunit is essential to the function of the AP2 complex. RNAi knockdown of α-adaptin causes mislocalization of clathrin and impaired endocytosis of transferrin (Motley *et al.*, 2003; Motley *et al.*, 2006). α-adaptin is a large (110-130 kDa) subunit composed of an amino-terminal trunk connected by a short linker to a carboxy-terminal ear domain. The trunk region, along with the trunk of the β2

subunit, provides a structural core for the complex. In addition, the trunk also binds to $PI(4,5)P_2$ (Gaidarov and Keen, 1999). This property is important for AP2 function, as transferrin uptake is impaired in α-adaptin mutants defective in $PI(4,5)P_2$ binding (Motley *et al.*, 2006). The ear domain of α-adaptin is divided into two structural subdomains: a platform flanked by 2 α-helices, and a β-sandwich. As discussed above, the ear domain of α-adaptin is important for binding a wide variety of other clathrin adaptors, underscoring the role of AP2 in organizing the network of proteins in a coated pit.

1.5.1.2 β2 subunit

β2-adaptin is similar in size and structure to α-adaptin, containing a trunk, hinge, and ear domain. As with α-adaptin, the ear domain consists of platform and sandwich subdomains that bind to other endocytic proteins. In addition, β2-adaptin binds to clathrin in the hinge region between the ear and the trunk (Shih *et al.*, 1995). β2-adaptin also plays a role in cargo selection. The transmembrane receptors TGF-β and GLUT8 have been shown to bind directly to the trunk of β2-adaptin at the plasma membrane (Schmidt et al., 2006; Yao et al., 2002).

1.5.1.3 μ2 subunit

The medium subunit μ2 binds to the central core of the AP2 complex. The binding site for μ2 is formed by the trunk domains of both α and β2 subunits, although the contact with β2 appears to be more extensive (Collins *et al.*, 2002). The primary function of μ2 is to recognize endocytic cargo, particularly cargo bearing a YXXΦ motif (Kirchhausen, 1999; Ohno et al., 1995).

In its native conformation, binding site for cargo is masked and affinity for YXXΦ is low (Collins *et al.*, 2002). But, when AP2 binds PI(4,5)P_2, μ2 changes conformation and exposes the cargo binding site (Rohde *et al.*, 2002). The μ2 subunit is also modified by phosphorylation, which increases its affinity for binding cargo (Olusanya et al., 2001; Pauloin and Thurieau, 1993; Ricotta et al., 2002).

1.5.1.4 σ2 subunit

The σ2 subunit of AP2 is the smallest of the four subunits. σ2 shares a high degree of sequence similarity with μ2 in the region where it binds to the trunk domains of α and β2 (Collins *et al.*, 2002). The σ2 subunit has not been shown to have any additional functionality beyond binding the other AP2 subunits, suggesting that its primary function is to stabilize the complex.

1.5.2 Epsin

Epsin is a clathrin adaptor first identified for its ability to bind eps15 (Chen *et al.*, 1998). While some epsin homologs, such as epsinR or enthoprotin, function at the trans-Golgi network (Drake *et al.*, 2000; Kalthoff *et al.*, 2002b), this discussion will focus on the function of epsin at the plasma membrane.

Like many clathrin adaptors, epsin has a modular organization. At the amino terminus, an ENTH domain interacts with the membrane by binding specifically to PI(4,5)P_2 (Itoh *et al.*, 2001). The carboxy-terminal region of epsin has little secondary structure; instead it contains multiple short binding motifs for clathrin, AP2, EH domain proteins, and ubiquitinated endocytic cargo. Each of these features is important for the function of epsin in the cell.

1.5.2.1 ENTH domain

The ENTH domain is a highly conserved globular domain composed of 7-8 compact α-helices. Structural comparisons indicate a high degree of structural similarities between the *armadillo* repeats of β-catenin, the HEAT repeat of karyopherin-β, and the VHS domain of human Tom1 (Hyman et al., 2000; Koshiba et al., 2002; Misra et al., 2000). These structures are most conserved along surfaces shown to be involved in protein-protein interactions.

Efforts to determine the function of the ENTH domain led to the surprising finding that the ENTH domain binds to the transcription factor PLZF (Hyman *et al.*, 2000). When crm-1-dependent nuclear export is blocked with leptomycin B treatment, epsin accumulates in the nucleus (Hyman *et al.*, 2000; Vecchi *et al.*, 2001). Interestingly, the clathrin adaptors CALM and eps15 also accumulate in the nucleus with leptomycin B treatment (Vecchi *et al.*, 2001). This suggests that clathrin accessory proteins may have a dual role at the plasma membrane and in the nucleus. Additionally, *liquid facets*, the fly homolog of epsin, genetically interacts with the nuclear protein *split ends* (Eun *et al.*, 2007). However, while eps15 and CALM induce transcription from a model promoter, epsin does not (Vecchi *et al.*, 2001). The function of the ENTH domain in the nucleus remains unknown.

A major breakthrough in the understanding of the ENTH domain came when Itoh and colleagues demonstrated that the ENTH domain binds specifically to $PI(4,5)P_2$ on the plasma membrane (Itoh *et al.*, 2001). This binding induces the formation of an α0-helix on the amino-terminal end of the ENTH domain (Ford *et al.*, 2002). This α0-helix inserts into the membrane, inducing curvature (Ford *et al.*, 2002; Kweon *et al.*, 2006). These findings led to a model for epsin function where epsin

binds to components of the clathrin coat and tethers them to the membrane via the ENTH domain, which also induces the curvature necessary for invagination.

There is some disagreement about whether the ENTH domain is required for epsin function. Dominant negative experiments using mammalian tissue culture cells have shown that overexpressing mutant epsin constructs that lack the ENTH domain causes a dominant negative effect on insulin, transferrin, and EGF receptor internalization, presumably by binding and sequestering clathrin and key clathrin adaptors (Chen et al., 1998; Ford et al., 2002; Itoh et al., 2001; Nakashima et al., 1999; Wang et al., 2006a). Mutant epsin constructs that no longer bind $PI(4,5)P_2$ have the same inhibitory effect (Itoh *et al.*, 2001). These data reinforce the importance of the ENTH domain to epsin function. However, these conclusions are complicated by the fact that overexpression of full-length, unmutated epsin can also interfere with endocytosis (Drake et al., 2000; Kariya et al., 2000; Morinaka et al., 1999). Additionally, these studies were done in the presence of endogenous epsin.

In yeast, only the ENTH domain is required to restore viability to the otherwise lethal epsin null mutants, and the carboxyl-terminal domain that contains only binding motifs does not rescue (Aguilar et al., 2003; Wendland et al., 1999). Temperature-sensitive mutations in key residues of the ENTH domain also cause significant endocytic defects (Wendland *et al.*, 1999). These results also reinforce an essential role for the ENTH domain. There are, however, important mechanistic differences between endocytosis in yeast compared to other eukaryotes. For example, AP2, which plays a major role in vertebrate endocytosis, appears to have limited function in endocytosis in yeast (Huang *et al.*, 1999). Accordingly, yeast epsins lack the AP2-binding motifs common to other epsins.

By contrast, in *Drosophila melanogaster,* both the ENTH domain and the carboxy-terminal domain with clathrin and adaptor binding motifs each rescue epsin deficiencies (Overstreet *et al.*, 2003). The ability of the ENTH-less fragment of epsin to rescue null mutants suggests that the ENTH domain is not required for epsin function in all organisms.

Recently, the ENTH domain was shown to have another function in addition to $PI(4,5)P_2$ binding. In yeast, the ENTH domain of the epsin homolog ent1p regulates cdc42 by binding to rga1p, a GAP for cdc42. Mutating residues involved in GAP binding prevented the ENTH domain from rescuing *ent1ent2*ΔΔ yeast cells (Aguilar *et al.*, 2006). Complementing these results is the finding that *Dictyostelium* epsin is required for the localization and function of the actin-binding adaptor Hip1r (Repass *et al.*, 2007). Thus the ENTH domain may play a role in regulating actin dynamics during clathrin-mediated endocytosis.

1.5.2.2 Interaction with ubiquitin via UIM

Many members of the epsin family contain UIMs (*u*biquitin *i*nteracting *m*otifs), which have the consensus sequence of ΦXXAXXXSXX*, where Φ is a bulky hydrophobic reside and * is an acidic residue (Hofmann and Falquet, 2001). These motifs have been shown to interact with ubiquitin and ubiquitinated cargo (Aguilar *et al.*, 2003; Miller *et al.*, 2004). Additionally, these motifs can also target epsin for ubiquitination (Miller et al., 2004; Oldham et al., 2002). Epsin genetically interacts with the deubiquitinating enzyme *fat facets* in flies (Cadavid *et al.*, 2000). The interaction between epsin and ubiquitin is important for epsin to localize to the membrane and function in yeast, where ubiquitination is a major player in endocytosis (Aguilar *et al.*, 2003).

In mammals, epsin does not require UIMs to localize to clathrin coated pits on the plasma membrane (Stang *et al.*, 2004), but epsin is required for the internalization of ubiquitinated cargo (Duncan et al., 2006; Sorkina et al., 2006; Stang et al., 2004). However, the mechanism by which epsin induces this internalization and the extent of ubiquitination required are matters of some debate.

The interaction between a single UIM and one ubiquitin is relatively weak, suggesting that multiple interactions are required for stable binding. The arguments for monoubiquitination as a sufficient signal for internalization stem from experiments using ubiquitin fusion proteins. Chimeric versions of the yeast plasma membrane protein ste2p and mammalian EGF receptor are quickly internalized when fused to ubiquitin (Haglund et al., 2003; Shih et al., 2000). Monoubiquitin:GFP that is targeted to the membrane by a lipid anchor and has been mutated to prevent polyubiquitination is internalized into the same endosomes as EGF (Chen and De Camilli, 2005). On the other hand, GST pull-down experiments with varying lengths of ubiquitin chains demonstrate that epsin selectively binds polyubiquitin (Hawryluk *et al.*, 2006). A CD4 chimera fused to ubiquitin was efficiently internalized when allowed to oligomerize, but was not internalized when mutated to prevent ubiquitin polymerization (Barriere *et al.*, 2006). These contrasting results may be reconciled by considering that in many instances, transmembrane proteins are ubiquitinated in multiple locations, leading to multimonoubiquitination (Haglund et al., 2003; Mosesson et al., 2003; Terrell et al., 1998). Other receptors, such as EGFR and PDGFR, must oligomerize in order to internalize (Sorokin et al., 1994; Westermark et al., 1989). Multimonoubiquitination and dimerization of monoubiquitinated cargo may mimic the binding of polyubiquitin to epsin UIMs, leading to more efficient internalization.

The canonical view of epsin function is that epsin promotes receptor internalization via clathrin-coated pits. This view was challenged by reports that association of epsin with ubiquitin excluded epsin from clathrin pits (Chen and De Camilli, 2005; Sigismund *et al.*, 2005). In the study by Chen and colleagues, depletion of clathrin by RNAi increased association of epsin with GFP-tagged ubiquitin at the plasma membrane. Mutation of the clathrin binding sites of epsin had the same effect (Chen and De Camilli, 2005). While these results were interpreted to mean that clathrin negatively regulated the association of epsin with ubiquitin, an alternative interpretation is that epsin targets ubiquitinated cargo to clathrin pits, and in the absence of clathrin there is an increase in the amount of epsin associated with uninternalized ubiquitinated cargo.

Epsin has also been proposed to promote endocytosis of ubiquitinated cargo by a caveolin-mediated pathway. Epsin preferentially co-precipitates with the ubiquitinated form of EGFR, and overexpression of EGFR leads to an increase in its ubiquitination and association with caveolin at the membrane. Mutant EGFR constructs that cannot be ubiquitinated colocalize only with clathrin pits at the membrane, while a chimeric EGFR-Ub construct colocalize with caveolin and not clathrin (Sigismund *et al.*, 2005). These results were contradicted by another study that did not find significant colocalization of epsin and caveolin under physiological conditions, nor did epsin redistribute to caveolin pits upon stimulation of EGFR (Hawryluk *et al.*, 2006). Inactive EGFR has been shown to internalize by a caveolin-dependent mechanism, while active EGFR is internalized via clathrin pits (Zhu *et al.*, 2005). This may account for the discrepancy in EGFR localization in cells overexpressing EGFR. Furthermore, internalization of a CD4-Ub chimera was completely blocked by RNAi-mediated reduction of clathrin heavy chain and by

known chemical inhibitor of clathrin-mediated endocytosis (Barriere *et al.*, 2006). Thus, the most likely scenario is that epsin promotes internalization of ubiquitinated cargo via a clathrin-dependent mechanism.

1.5.2.3 Interaction with clathrin and other adaptors

While some have suggested epsin may function in a clathrin independent manner (see above), the predominant view is that epsin functions at clathrin-coated pits. All epsins contain clathrin-binding motifs that bind to the terminal domain of clathrin heavy chain (Drake *et al.*, 2000). Epsin colocalizes with clathrin and other clathrin adaptors on the plasma membrane, and is present, but not enriched, in purified clathrin-coated vesicles (Chen *et al.*, 1998). In flies, epsin genetically interacts with clathrin heavy chain (Cadavid *et al.*, 2000), and mutating epsin disrupts clathrin-mediated endocytosis in many systems (Overstreet et al., 2003; Song et al., 2006; Wang et al., 2006a). Epsin also promotes clathrin assembly (Kalthoff *et al.*, 2002a).

Most epsins, with the notable exception of yeast, contain two or more DPF/W motifs for binding the α-ear of AP2 (Owen *et al.*, 1999; Traub *et al.*, 1999). As with other short binding motifs, the interaction between a single DPF/W and AP2 is relatively weak, and multiple interactions are required to form a stable network *in vivo*. The AP2-binding motifs of epsin may cooperate with the clathrin-binding motifs to stabilize each other at the membrane (Drake *et al.*, 2000). There is also some evidence that epsin binds to the β-ear of AP2, facilitating the formation of an interconnected network of adaptors in a coated pit (Owen *et al.*, 2000). Epsins also contain NPF motifs that bind specifically to the EH domains of adaptors such as eps15. While this interaction led to the initial identification of epsin (Chen *et al.*,

1998), little additional work has been done to determine the importance of the interaction of epsin and EH domains in clathrin-coated pit formation.

1.5.2.4 Epsin is an adapting adaptor

To fully understand how epsin functions in the cell, it is necessary to step back from specific interactions and study how epsin affects the endocytic process as a whole. RNAi-mediated knockdown experiments have shown that the reduction of epsin in the cell affects the internalization of different receptors than the knockdown of AP2 (Barriere *et al.*, 2006), suggesting that the requirements for these two adaptors varies depending on the circumstances. Epsin may be best suited to a subset of clathrin events.

Epsin is particularly important for the Notch/Delta signaling pathway in both *Drosophila* and *C. elegans* (Overstreet *et al.*, 2003; Overstreet *et al.*, 2004; Tian *et al.*, 2004). In this pathway, the internalization of Delta ligand in the signaling cell is important for the activation of Notch in the receiving cell. Surprisingly, the loss of epsin does not affect bulk endocytosis of Delta ligand (Wang and Struhl, 2005). However, only Delta ligands internalized by an epsin-dependent pathway are able to activate Notch (Wang and Struhl, 2004; Wang and Struhl, 2005). This suggests that epsin may have more than a generic role in clathrin-mediated endocytosis. As a clathrin adaptor, epsin may selectively adapt clathrin machinery to process certain endocytic events in a specific manner.

1.5.3 Hip1r

Hip1r is a unique clathrin adaptor in that it binds to clathrin, the plasma membrane, and actin, actins as a molecular bridge between clathrin and the actin cytoskeleton (Engqvist-Goldstein *et al.*, 2001; Newpher and Lemmon, 2006). In yeast, other actin-associated endocytic components, such as the WASP protein Bbc1, are recruited to the coated pit just before scission. However Hip1r appears earlier, arriving at endocytic sites shortly after clathrin (Kaksonen et al., 2003; Kaksonen et al., 2005; Newpher and Lemmon, 2006). Hip1r function is closely tied to its structural organization. At the amino terminus, Hip1r contains an ANTH domain, followed by a coiled-coil region. The carboxy-terminus contains an actin-binding THATCH domain. We will consider the functions of each of these regions in turn.

1.5.3.1 ANTH domain

The ANTH domain of Hip1r binds to $PI(4,5)P_2$ on the plasma membrane, similar to other ANTH domains (Sun *et al.*, 2005). However, when expressed in mammalian cells, the ANTH domain alone was diffuse in the cytosol (Engqvist-Goldstein *et al.*, 1999). This may be an indication that the ANTH domain binds $PI(4,5)P_2$ weakly or transiently. The interactions on the rest of Hip1r may be necessary to stabilize Hip1r at the membrane. The ANTH domain of Hip1r is also involved in actin-mediated processes during endocytosis. Although the ANTH domain does not bind to actin, mutations in the ANTH domain lead to disorganized cortical actin in yeast (Sun et al., 2005; Wesp et al., 1997). The ANTH domain may influence actin organization by properly orienting the actin-binding THATCH domain of Hip1r at the plasma membrane (Sun *et al.*, 2005).

1.5.3.2 Coiled-coil domain

The central coiled-coil region of Hip1r mediates dimerization and is important for binding to clathrin at the plasma membrane (Engqvist-Goldstein et al., 2001; Legendre-Guillemin et al., 2005; Wesp et al., 1997). These two functions work together to allow to Hip1r promote clathrin assembly (Chen and Brodsky, 2005; Legendre-Guillemin et al., 2002; Legendre-Guillemin et al., 2005). Most clathrin adaptors bind to clathrin heavy chain, making the ability of Hip1r to bind clathrin light chain unique. This interaction is not mediated by short peptide motifs as in other adaptors, but rather the coiled coil region forms a hydrophobic groove that binds near the amino terminus of clathrin light chain (Chen and Brodsky, 2005; Ybe *et al.*, 2007a). This is the same region of clathrin light chain that binds to the hub region of clathrin heavy chain (Liu *et al.*, 1995; Ybe *et al.*, 1998). The coiled-coil domain of Hip1r is sufficient to localize to clathrin puncta on the membrane, although it is unclear whether this is a result of clathrin binding alone or dimerization of the truncation with endogenous full-length Hip1r (Engqvist-Goldstein *et al.*, 2001).

1.5.3.3 THATCH: Hip1r's unique regulation of actin

At its extreme carboxy-terminus, Hip1r contains a THATCH domain (*t*alin-*H*IP1/R/Sla2p *a*ctin-*t*ethering *C*-*t*erminal *h*omology), also known as an I/LWEQ module. This domain binds to F-actin and is found on many proteins that regulate actin assembly (Engqvist-Goldstein et al., 1999; Hemmings et al., 1996; McCann and Craig, 1997; McCann and Craig, 1999). The THATCH domain forms a cylindrical core of five anti-parallel α-helices, with α3 and α4 forming a highly conserved actin-binding patch along one face (Brett *et al.*, 2006). THATCH domains also dimerize via an additional helix C-terminal to the core structure (Brett *et al.*, 2006; Smith and

McCann, 2007). This dimerization increases affinity for actin and promotes formation of stable F-actin filaments *in vitro* (Brett et al., 2006; Senetar et al., 2004).

The first indication that Hip1r was important in actin dynamics came when the yeast homolog, sla2p, was found to be synthetic lethal with the actin-binding protein abp1p (Holtzman *et al.*, 1993). Sla2p mutants have disorganized cortical actin and are defective in endocytosis (Holtzman *et al.*, 1993; Raths *et al.*, 1993; Yang *et al.*, 1999). Clathrin-coated pits do not properly mature in sla2p mutants, but instead remain immobilized on the membrane. This phenotype mimics the phenotype of cells treated with the actin-depolymerizing drug Latrunculin A (Kaksonen *et al.*, 2003; Newpher and Lemmon, 2006). A closer look at clathrin in sla2p mutant cells reveals the formation of small, non-functional actin comets near the site of clathrin assembly (Kaksonen *et al.*, 2003). Such bursts of actin polymerization without internalization of the clathrin pit are also observed in RNAi-treated mammalian cells with reduced levels of Hip1r (Engqvist-Goldstein *et al.*, 2004). This suggests that Hip1r is responsible for the productive coupling of actin dynamics to the coated pit (Kaksonen *et al.*, 2003).

A possible mechanism for this regulation could be an interaction between Hip1r and cortactin. RNAi targeting of cortactin partially suppresses the phenotypes induced by the knockdown of Hip1r (Engqvist-Goldstein *et al.*, 2004). The SH3 domain of cortactin binds to a proline-rich area of Hip1r. Hip1r and cortactin together inhibit actin polymerization, and Hip1r prevents the association of cortactin with dynamin (Le Clainche *et al.*, 2007). This data leads to a model where Hip1r, which associates with clathrin for the duration of coated pit assembly, prevents the inappropriate polymerization of actin until the pit is fully mature and ready for scission.

1.6 STUDYING CLATHRIN PROCESSES IN *DICTYOSTELIUM DISCOIDEUM*

The social amoeba *Dictyostelium discoideum* is an excellent eukaryotic model system for studying membrane traffic. *Dictyostelium* has a haploid genome that is highly amenable to genetic manipulation. *Dictyostelium* cells are large enough to be practical for microscopy, and can be grown in large quantities for biochemical studies. In addition, this lab has identified, cloned, tagged, and expressed a wide array of endocytic proteins, including α-adaptin, β2-adaptin, AP180, epsin, Hip1r, auxilin, and clathrin light and heavy chains. Null mutants of these proteins have also been generated and characterized. This provides many tools to investigate the function of specific adaptors and how they contribute to clathrin processes in the cell.

1.7 GOALS OF MY DOCTORAL WORK

The main goals of my dissertation were to identify determinants for epsin localization and function within the cell and to address the significance of the interaction between epsin and Hip1r. To do so, I identified and characterized EpnA, the *Dictyostelium* homolog of human Epsin1. I found that *Dictyostelium* epsin was not required for global clathrin function, but played an essential role in spore development. I demonstrated that clathrin, but not AP2, was critical for epsin to associate with clathrin-coated pits. Furthermore, the carboxy-terminal region of epsin is essential, but not sufficient, for targeting epsin within clathrin-coated pits on the plasma membrane. This carboxy-terminal domain must be coupled to the ENTH domain: the carboxy-terminal domain alone associates with intracellular clathrin, but is excluded from the plasma membrane. In addition to targeting epsin to the

membrane, the amino-terminal ENTH domain regulates the interaction between epsin and clathrin, an essential property that cannot be replaced by an alternate $PI(4,5)P_2$ binding domain. Within the ENTH domain, residues important for PIP2-binding were essential for both epsin localization and function, while residue T107 was essential for function but not coated pit localization. My results support a model where the ENTH domain coordinates with the clathrin-binding C-terminal domain to allow a dynamic interaction of epsin with coated pits.

My work in investigating the relationship between epsin and Hip1r revealed that epsin influenced clathrin and actin dynamics at the plasma membrane. I determined that small bursts of actin polymerization accompany the internalization of clathrin puncta from the plasma membrane in *Dictyostelium*. In epsin null cells, clathrin-coated pits persisted much longer at the plasma membrane, and many transient actin puncta developed without associating with clathrin puncta. Epsin is required for the phosphorylation and membrane recruitment of Hip1r. The deficiencies in clathrin and actin dynamics of epsin null cells were more pronounced in Hip1r null cells, indicating that Hip1r also influences these processes. I propose that epsin facilitates the productive coupling of actin with clathrin-coated pits by regulating the activity Hip1r. Taken together, my results demonstrate the cooperative and dynamic nature of adaptor proteins during clathrin-mediated endocytosis.

Chapter 2: Cooperation between ENTH and Carboxy-terminal Domains of Epsin Regulates Dynamic Interaction with Clathrin-Coated Pits in *Dictyostelium*

2.1 INTRODUCTION

Clathrin-mediated endocytosis is a highly conserved process in which specific cargo on the plasma membrane is selected and internalized. Clathrin triskelia, key structural proteins of this process, are recruited to the membrane and assemble into coated pits that encompass endocytic cargo. These pits subsequently pinch off to form intracellular clathrin-coated vesicles. A wide variety of adaptors and accessory proteins select appropriate cargo and help recruit clathrin to the membrane (Owen *et al.*, 2004; Robinson, 2004; Sorkin, 2004). Epsin is one such clathrin adaptor.

At the amino-terminus, epsin contains an ENTH domain (Epsin N-Terminal Homology) that binds specifically to the lipid PtdIns(4,5)P_2 (Itoh *et al.*, 2001). At the carboxy-terminus, epsin contains several short binding motifs specific for clathrin and clathrin adaptors such as AP2 and EH-domain proteins (Chen *et al.*, 1998; Kay *et al.*, 1999). This modular organization suggests a model where the carboxy-terminus of epsin acts as a scaffold for clathrin, clathrin adaptors, and specific cargo, while the amino-terminal ENTH domain tethers and promotes invagination of the coated pit from the plasma membrane. However, how these modules cooperate to facilitate epsin function in living cells remains unclear. Furthermore, domain analysis of fly and yeast epsin has led to the puzzling result that expression of just the ENTH domain rescues phenotypic deficiencies in these organisms, suggesting that the carboxy-terminus is dispensable (Aguilar et al., 2003; Overstreet et al., 2003; Wendland et al., 1999), The capacity of the isolated ENTH domain to function raises

questions about what functional properties the carboxy-terminal domain contributes to epsin.

Dictyostelium cells offer a model system where clathrin-coated pits associate with the plasma membrane, and clathrin is essential for important biological roles (O'Halloran and Anderson, 1992; Damer and O'Halloran, 2000; Wang et al., 2006). Moreover, *Dictyostelium* cells contain conserved adaptors that associate with clathrin on the plasma membrane (Repass et al., 2007; Stavrou and O'Halloran, 2006; Wang et al., 2006b). In this study, we identified the *Dictyostelium* epsin ortholog, epnA, and found that it plays an essential role in spore development. In addition, our analysis highlighted separate and distinct contributions of the ENTH domain and the carboxy-terminal domain to the localization and to the functional capacity of epsin. We conclude that the ENTH domain cooperates with the C-terminal domain of epsin to facilitate a dynamic interaction with clathrin-coated pits at the plasma membrane.

2.2 RESULTS

2.2.1 Identification of *Dictyostelium* epsin

By searching for genes that shared amino acid sequences similar to the ENTH domain of human Epsin1, we identified the *Dictyostelium discoideum* ortholog of epsin from the *Dictyostelium* genome database (see Appendix A: Materials and Methods). We identified a single gene, which we named *epnA*, with high amino acid sequence identity (48%) to the Epsin1 ENTH domain. This was the sole gene that contained an ENTH domain; an EpsinR gene was not identified. From this we concluded that *Dictyostelium* contains a single gene for epsin.

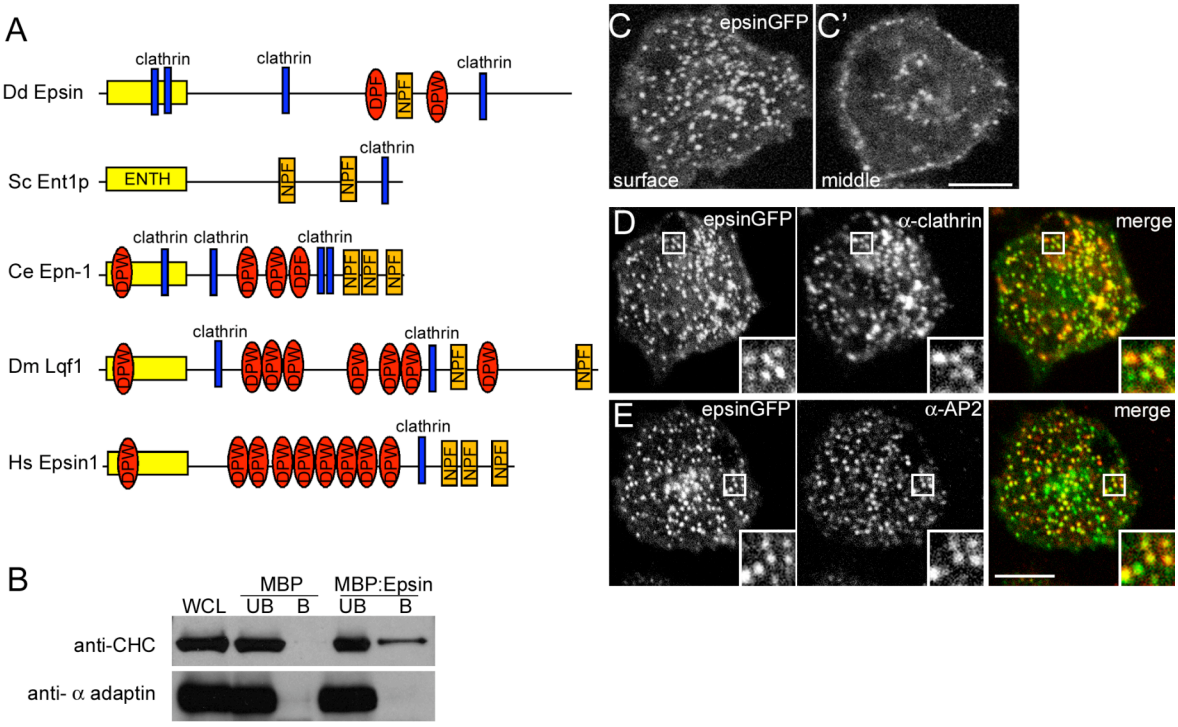

Figure 2.1. *Dictyostelium* epsin.

(A) Schematic representation of the structural organization of epsins from *Dictyostelium* (Dd), *S. cerevisae* (Sc), *C. elegans* (Ce), *Drosophila* (Dm), and *Homo sapiens* (Hs). Each has a membrane binding ENTH domain (yellow box) as well as motifs for binding clathrin (blue boxes), a DPF/DPW motif that binds AP-2 (red oval) and an NPF for binding EH domain-containing proteins. (B) Epsin coprecipitates with clathrin. Bacterially expressed MBP alone or MBP:Epsin was bound to amylose resin and incubated with cleared *Dictyostelium* lysate. Unbound (UB) and bound (B) fractions were immunoblotted for clathrin (anti-CHC) or alpha adapton (anti-α adaptin).(C) Confocal image of a focal plane from the surface of a wild-type cell expressing epsin:GFP. (C') Confocal image of a focal plane from the middle of the same cell. (D and E) Epsin colocalizes extensively with clathrin and AP2. Surface views of confocal images of cells expressing epsin:GFP (green) and immunostained for (D) clathrin or (E) AP2 (red). Bar, 5μm.

The predicted amino acid sequence for *epnA* contained multiple short binding motifs for other endocytic adaptors (Figure 2.1A), consistent with epsins from other species (Cadavid et al., 2000; Chen et al., 1998; Kay et al., 1999; Salcini et al., 1997; Traub et al., 1999). In addition, *Dictyostelium* epsin also contained two Type I L(L,I)(D,E,N)(L,F)(D,E,S) clathrin-binding motifs (Dell'Angelica *et al.*, 1998; Drake *et al.*, 2000; ter Haar *et al.*, 2000) and two clathrin-binding (D/S)LL motifs (Morgan *et al.*, 2000) (Figure 2.1A). However, unlike most epsins in other species, the predicted amino acid sequence for *Dictyostelium* epsin did not contain a ubiquitin-interacting motif (Aguilar et al., 2003; Barriere et al., 2006; Hofmann and Falquet, 2001; Polo et al., 2002). In this respect, *Dictyostelium* epsin was similar to *Arabadopsis* epsin, which also lacks a ubiquitin-interacting motif (Holstein and Oliviusson, 2005). To confirm the ability of epsin to bind to clathrin, we performed a pulldown binding assay. Bacterially-expressed MBP:epsin (*m*altose-*b*inding *p*rotein) fusion protein was bound to amylose resin and incubated with *Dictyostelium* cell lysate. Analysis of the bound and unbound fractions revealed that clathrin coprecipitated with MBP:epsin, but not MBP alone (Figure 2.1B). Under these conditions, we were not able to detect binding between epsin and AP2 (Figure 2.1B).

To determine the cellular location of *Dictyostelium* epsin, we cloned a cDNA for *epnA* fused to GFP (Green Fluorescent Protein) and expressed this epsin:GFP fusion construct in a wild-type background. As with mammalian epsins (Chen *et al.*, 1998), *Dictyostelium* epsin showed a punctate distribution largely restricted to the plasma membrane, with some intracellular puncta (Figure 2.1C). The epsin puncta colocalized with clathrin on the plasma membrane and also with intracellular clathrin puncta (Figure 2.1D, Figure 2.5B). *Dictyostelium* epsin puncta also colocalized extensively with AP2 at the plasma membrane (Figure 2.1E, Figure 2.5A). Thus both

the domains and localization of *Dictyostelium* epsin are similar to epsins from other organisms.

2.2.2 Epsin null mutants display limited clathrin-associated phenotypes and have abnormal spore morphology

To examine the contribution of *Dictyostelium* epsin to cellular functions, we used targeted gene replacement to generate two epsin null mutants. The deletion of the *epnA* gene in these mutants was confirmed by PCR (Polymerase Chain Reaction) of genomic DNA (Appendix D, Figure D.1), and the absence of epsin protein expression was demonstrated by immunoblotting with anti-epsin antibodies (Figure 2.2A). Subsequent experiments revealed no differences in phenotype between the two epsin null cell lines.

Reconstitution experiments with purified proteins and liposomes suggest that epsin functions to invaginate clathrin-coated pits (Ford et al., 2002). If epsin contributes this essential role to clathrin-coated vesicle formation in living cells, epsin null cells would be expected to exhibit clathrin-related phenotypic deficits. To test whether clathrin-mediated cellular functions were compromised by the loss of epsin, we assessed the epsin null mutants for phenotypes displayed by clathrin mutants. These phenotypes include defects in osmoregulation in hypo-osmotic conditions, deficiencies in fluid-phase endocytosis, and abnormal development into fruiting bodies (Niswonger and O'Halloran, 1997a; O'Halloran and Anderson, 1992; Wang et al., 2003). All of these processes were normal in the epsin null mutants (Figure 2.2B and Appendix D, Figure D.2), suggesting that epsin is not critical for general clathrin function. Both clathrin heavy chain null (chc-) and clathrin light chain null (clc-) mutants are known to fail in cytokinesis when grown in suspension cultures

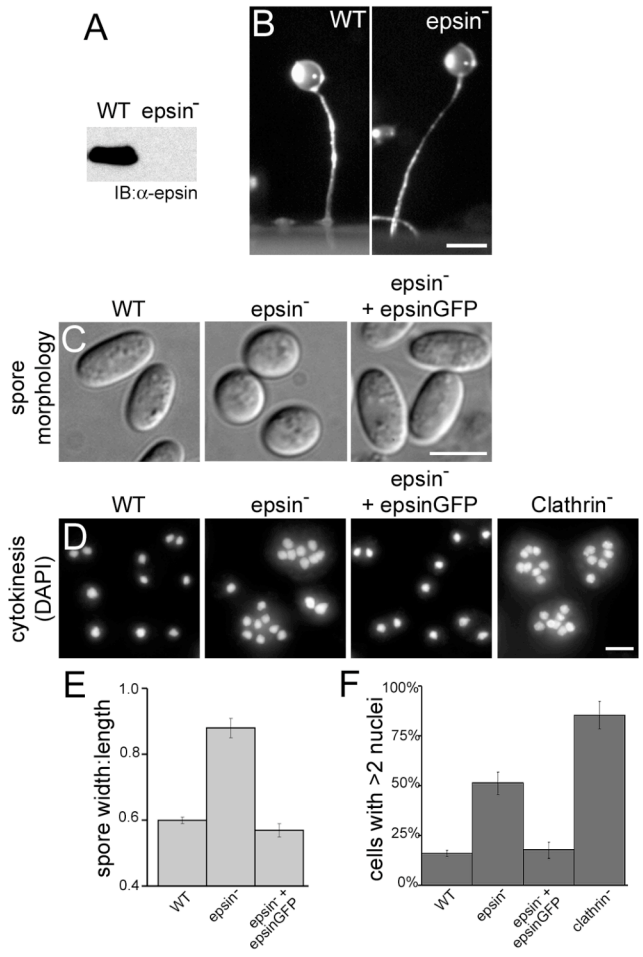

Figure 2.2. Epsin-null mutants display defects in cytokinesis and spore morphology.

(A) An immunoblot of wildtype (WT) and epsin-null mutants (epsin$^-$) stained with anti-epsin antibodies. (B) Wild-type (WT) and epsin mutants (epsin$^-$) develop into fruiting bodies. Bar, 0.2mm. (C) Wild-type (WT), epsin-null mutants (epsin$^-$), clathrin-null mutants (clathrin$^-$), and epsin-null mutants expressing epsin:GFP (epsin$^-$ +epsin:GFP) grown in suspension for 72hrs and stained with DAPI to visualize nuclei. Bar, 5μm. (D) DIC images of spores harvested from fruiting bodies of wild-type cells (WT), epsin-null mutants (epsin$^-$), and epsin-null mutants expressing epsin:GFP (epsin$^-$ +epsin:GFP). Expression of epsin:GFP restores wild-type sore morphology. Bar, 5μm. (E) Ratio of spore length:width in wild-type (WT), n=50; epsin null mutants (epsin-), n=50; and epsin null mutants expressing epsin:GFP (epsin$^-$+epsinGFP), n=50. (F) Quantification of multinucleated cells in suspension cultures of wildtype (WT), epsin mutants (epsin$^-$), epsin mutants expressing epsin:GFP (epsin$^-$+epsin:GFP), and clathrin heavy chain mutants (clathrin$^-$); n=300 for each cell line. Error bars are standard error.

(Niswonger and O'Halloran, 1997a; Wang *et al.*, 2003). Similarly to the clathrin mutants, epsin null cells also accumulated multiple nuclei when grown in suspension cultures (Figure 2.2, D and F). The absence of many phenotypes characteristic of clathrin mutants suggested that epsin does not supply an essential and global function, such as invagination, to clathrin-coated pit formation. Rather the discrete phenotype suggests that epsin contributes to a subset of clathrin function that includes cytokinesis.

In contrast with clathrin null cells, epsin null mutants developed normally into fruiting bodies (Figure 2.2B). However, we noted an abnormal phenotype when examining the morphology of spores within mature fruiting bodies. Spores from wild-type fruiting bodies were oblong, but spores from epsin null fruiting bodies were round (Figure 2.2, C and E). Measurement of the width:length ratio of wild-type spores was 0.60 ± 0.01 (n=50; mean ± SE),whereas spores derived from epsin null mutants had a width:length ratio of 0.88 ± 0.02 (n=50; mean ± SE; Figure 2.2D and F). This round spore phenotype was reminiscent of *Dictyostelium* Hip1r, another clathrin accessory protein (Repass *et al.*, 2007). The restricted phenotype during development supported an essential role for epsin in a specialized pathway that controls the correct morphology of spores.

2.2.3 Clathrin and AP2 assemble into puncta on the membrane of epsin null cells.

Epsins contain domains and motifs that bind plasma membrane lipids as well as clathrin and clathrin adaptors. We therefore tested whether epsin was essential for clathrin pit organization by assessing the ability of clathrin and the clathrin adaptor AP2 to assemble into puncta on the plasma membrane of epsin null cells. Wild-type

and epsin null mutants were transformed with GFP-clathrin light chain, a marker known to reflect the endogenous distribution of clathrin (Wang *et al.*, 2006b), and then were immunostained with antibodies against AP2. In wild-type cells, clathrin formed puncta on the plasma membrane and in the cytoplasm (Figure 2.3A). Clathrin puncta on the plasma membrane of wild-type cells colocalized extensively with AP2 (Figure 2.3A, inset). In epsin null cells, clathrin and AP2 puncta also formed, and the frequency and distribution of the two proteins were indistinguishable from wild-type cells (Figure 2.3B). Subcellular fractionation of wild-type cells showed that clathrin partitioned into the low speed and the high speed membrane fractions. Clathrin showed a similar association with membrane fractions in epsin null cells (Figure 2.3C). Together, these observations suggested that epsin does not play an essential role in organizing clathrin or AP2 in coated pits.

2.2.4 Epsin localization into puncta on the plasma membrane requires clathrin

To address whether clathrin is required for the association of epsin with the plasma membrane, we examined the distribution of epsin tagged with GFP in clathrin null and AP2 null mutants. Both the cytokinesis and spore morphology defects of epsin mutants were completely rescued by expression of epsin:GFP (Figure 2.2, C-F), demonstrating that epsin:GFP was functional, and that the deficiencies displayed by epsin null cells were specific for the absence of epsin. In clathrin heavy chain mutants, clathrin-coated pits are absent (O'Halloran and Anderson, 1992). Likewise, epsin:GFP did not cluster into puncta in clathrin heavy chain mutants, but instead uniformly decorated the plasma membrane (Figure 2.3D). In *Dictyostelium* cells that

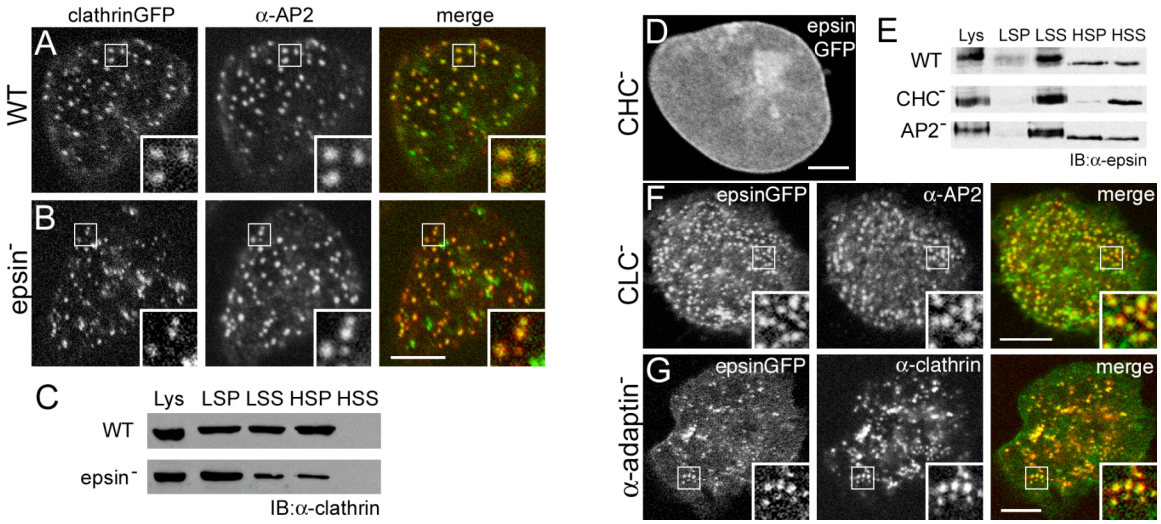

Figure 2.3. Epsin requires clathrin heavy chain to form puncta on the membrane.

(A and B) Clathrin and AP2 localization in epsin mutants is comparable to wild-type. Confocal images of (A) wild-type (WT) and (B) epsin-null mutants (epsin$^-$) expressing clathrin:GFP (green) and immunostained for AP2 (red). (C) Subcellular fractionation of clathrin is similar in wild-type (WT) and epsin-null cells (epsin$^-$). Cells were harvested, lysed, and centrifuged at 3000x*g* for 5min. Supernatants were centrifuged at 100,000x*g* for 1 hour. Immunoblot of samples probed with α-clathrin heavy chain antibodies. (Lys) lysate, (LSP) low-speed (3000x*g*) pellet, (LSS) low-speed supernatant, (HSP) High-speed (100,000x*g*) pellet, (HSS) high-speed supernatant. (D) Epsin localizes to the membrane but does not form puncta in clathrin heavy chain null cells. Confocal image of clathrin heavy chain null cells (CHC$^-$) expressing epsin:GFP (E) Subcellular fractionation of epsin is altered in clathrin heavy chain mutants, but not in α-adaptin-null mutants. (WT) wild-type, (CHC$^-$) clathrin heavy chain null, (AP2$^-$) α-adaptin null. Immunoblot probed with α-epsin antibodies. (F) Epsin forms puncta that colocalize with AP2 on the membranes of clathrin light chain mutants. Confocal images of clathrin light chain null mutants expressing epsin:GFP (green) and immunostained for AP2 (red). (G) Epsin forms reduced numbers of puncta at the plasma membrane in α-adaptin-null mutants that colocalize with clathrin. Confocal images of α-adaptin mutants expressing epsin:GFP (green) and immunostained for clathrin (red). Bars, 5μm.

lack clathrin light chain, clathrin function is diminished, but the heavy chain remains assembled into puncta on the plasma membrane (Wang *et al*., 2003). In these clathrin light chain mutants, epsin:GFP distributed into puncta on the plasma membrane and colocalized with AP2 (Figure 2.3F). These observations suggested that clathrin heavy chain influences the distribution of epsin on the plasma membrane. Subcellular fractionation studies of epsin confirmed this influence. In wild-type cells, epsin fractionated with the membranes of the high speed pellet. In contrast, epsin was found in the soluble high speed supernatant in clathrin heavy chain mutants (Figure 2.3E).

2.2.5 Epsin does not require AP2 to associate with clathrin at the plasma membrane

The preceding experiments established that clathrin was an important determinant for the association of epsin with membranes and for clustering within puncta on the plasma membrane. In addition to motifs for binding clathrin, the carboxy-terminus of epsin has motifs for binding AP2, the predominant and best-characterized clathrin adaptor at the plasma membrane. To examine the contribution of AP2 to the cellular location of epsin, we expressed epsin:GFP in AP2-alpha mutants lacking the large alpha subunit of AP2. Relative to wild-type cells, AP2-alpha mutants show reduced numbers of clathrin puncta on the plasma membrane (Wen and O'Halloran, unpublished results). Nonetheless, epsin continued to colocalize with the remaining clathrin puncta in AP2-alpha null cells (Figure 2.3G). Similar to the reduced number of clathrin puncta on the membrane of AP2-alpha mutants, epsin formed ~20% fewer puncta on the plasma membrane of AP2-alpha mutants (0.60 ± 0.04 puncta per μm^2, n=1047 puncta; 16 cells) compared to wild-type cells (0.77 ± 0.04 puncta per μm^2, n=683 puncta; 12 cells) (Figure 2.3G). Subcellular

fractionation of epsin in the AP2-alpha mutants revealed that the association of epsin with membrane fractions was similar to that seen in wild-type cells (Figure 2.3E). Taken together, these results indicate that, while AP2-alpha is important for building clathrin-coated pits on the plasma membrane, AP2-alpha is not a critical determinant for localizing epsin into coated pits.

2.2.6 The ENTH domain is required but is not sufficient for epsin association with clathrin and AP2 at the plasma membrane

In other organisms, the amino-terminal ENTH domain of epsin has been shown to be sufficient for phenotypic rescue (Wendland et al., 1999; Aguilar et al., 2003; Overstreet et al., 2003). To explore the functional properties of *Dictyostelium* epsin in more detail, we generated two expression plasmids for GFP-tagged epsin truncations, epsin$_{1\text{-}333}$ and epsin$_{253\text{-}677}$, that separated the amino-terminal ENTH domain from the carboxy-terminal domain which contained motifs for binding clathrin, EH-domain proteins, and AP2 (Figure 2.4).

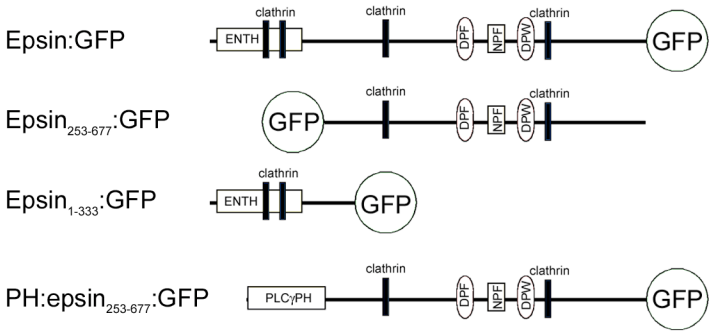

Figure 2.4. Schematic representation of GFP-labeled epsin truncation and chimera constructs.

Black bars indicate clathrin binding motifs, ovals indicate DPF/W AP2 binding motifs, and rectangles indicate NPF motifs for binding EH domain-containing proteins.

We expressed these truncation constructs in an epsin null background (Figure 2.5, C-F). Because the C-terminal domain of epsin contains motifs for binding AP2 and clathrin accessory proteins, we expected epsin$_{253-677}$ to associate with the plasma membrane. However, examination by fluorescence microscopy of cells expressing epsin$_{253-677}$ revealed that epsin$_{253-677}$:GFP rarely localized to the plasma membrane, but instead associated with puncta in the cytoplasm (Figure 2.5, C and D, compare with A and B). Thus the carboxy-terminal domain of epsin associated only with cytoplasmic puncta of clathrin and was excluded from plasma membrane clathrin puncta, contrary to the normal distribution for full-length epsin.

The complementary amino-terminal epsin construct, epsin$_{1-333}$, contained the complete ENTH domain plus a short, unstructured region. Consistent with the capacity of the ENTH domain to bind PI(4,5)P_2 , Epsin$_{1-333}$:GFP localized uniformly on the plasma membrane and did not form discrete puncta (Figure 2.5, E and F). Epsin$_{1-333}$:GFP also distributed along the plasma membrane of clathrin heavy or light chain null cells as well as AP2-alpha null cells, suggesting that the ability of epsin$_{1-333}$:GFP to associate with the plasma membrane was independent of clathrin or AP2 (Appendix D, Figure D.5). While the distribution of epsin$_{1-333}$ was uniform, clathrin localized normally into puncta in both epsin null and wild-type cells expressing epsin$_{1-333}$:GFP (Figure 2.5F and Appendix D, Figure D.4).

In addition to their localization, we also tested whether the amino-terminal and the carboxy-terminal truncations of epsin were able to rescue the phenotypic deficiencies of epsin null cells. We tested the ability of both constructs to rescue cytokinesis by examining whether multinucleated cells accumulated in cultures of epsin mutants expressing either of the two constructs. Quantification of multi-

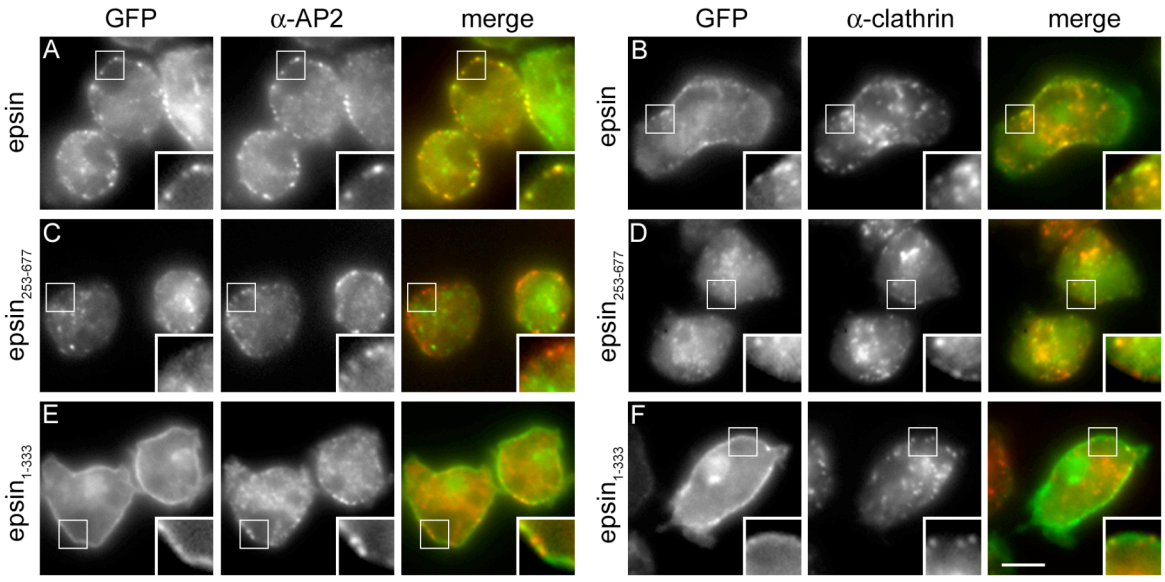

Figure 2.5. Both domain of epsin are required for targeting to clathrin-coated pits.

(A and B) Epsin:GFP colocalizes with AP2 in epsin null cells. Epsin null cells expressing epsin:GFP (green) were fixed and immunostained with anti-alpha-adaptin antibody (A) or anti-clathrin antibody (B) (red). (C) Epsin$_{253-677}$:GFP does not form puncta at the plasma membrane and does not colocalize with AP2. Epsin null cells expressing epsin$_{253-677}$:GFP (green) were fixed and immunostained with anti-alpha-adaptin antibody (red). (D) Epsin$_{253-677}$:GFP cytoplasmic puncta overlap with clathrin puncta in the cytoplasm but not the plasma membrane. Epsin null cells expressing epsin$_{253-677}$:GFP (green) were fixed and immunostained with anti-clathrin antibody (red). (E and F) Epsin$_{1-333}$:GFP uniformly decorates the plasma membrane. Epsin null cells expressing epsin$_{1-333}$:GFP (green) were fixed and immunostained with anti-alpha-adaptin antibody (E) or anti-clathrin antibody (F) (red). Bar, 5μm.

nucleated cells in suspension cultures revealed that both epsin$_{253-677}$:GFP and epsin$_{1-333}$:GFP rescued the cytokinesis defect of epsin mutants (Figure 2.6C).

We also examined the ability of the two constructs to rescue the spore morphology defect of epsin mutants. Epsin null cells expressing epsin$_{253-677}$:GFP developed into fruiting bodies containing round spores that were indistinguishable from the epsin null mutants (Figure 2.6, A and B). The failure of epsin$_{253-677}$:GFP to restore normal spore morphology was not an artifact of the GFP tag, because epsin null cells expressing epsin$_{253-677}$ without GFP also developed into fruiting bodies that contained round spores (Figure 2.6B). In contrast, epsin null mutants expressing the amino-terminal construct, epsin$_{1-333}$:GFP, developed into fruiting bodies that contained oblong spores indistinguishable from wild-type (Figure 2.6, A and B). Thus epsin$_{253-677}$:GFP was able to rescue the cytokinesis failure but not the spore morphology defect, while epsin$_{1-333}$ was able to fully rescue both phenotypic defects of epsin null mutants.

2.2.7 A canonical PI(4,5)P_2-binding domain cannot substitute for the ENTH domain

The analysis of epsin domains suggested that the ENTH domain was both necessary and sufficient to target epsin to the membrane and to rescue the spore morphology and cytokinesis defects of epsin null mutants. A significant function of the epsin ENTH domain is to bind PI(4,5)P_2 (Ford et al., 2002; Itoh et al., 2001). We therefore asked whether another PI(4,5)P_2-binding domain could functionally replace the ENTH domain of *Dictyostelium* epsin. The PH domain of mammalian PLCδ, a canonical PI(4,5)P_2-binding domain, is of comparable size to the ENTH domain and also binds to PI(4,5)P_2 (Itoh et al., 2001; Lemmon et al., 1995; Stauffer et al., 1998).

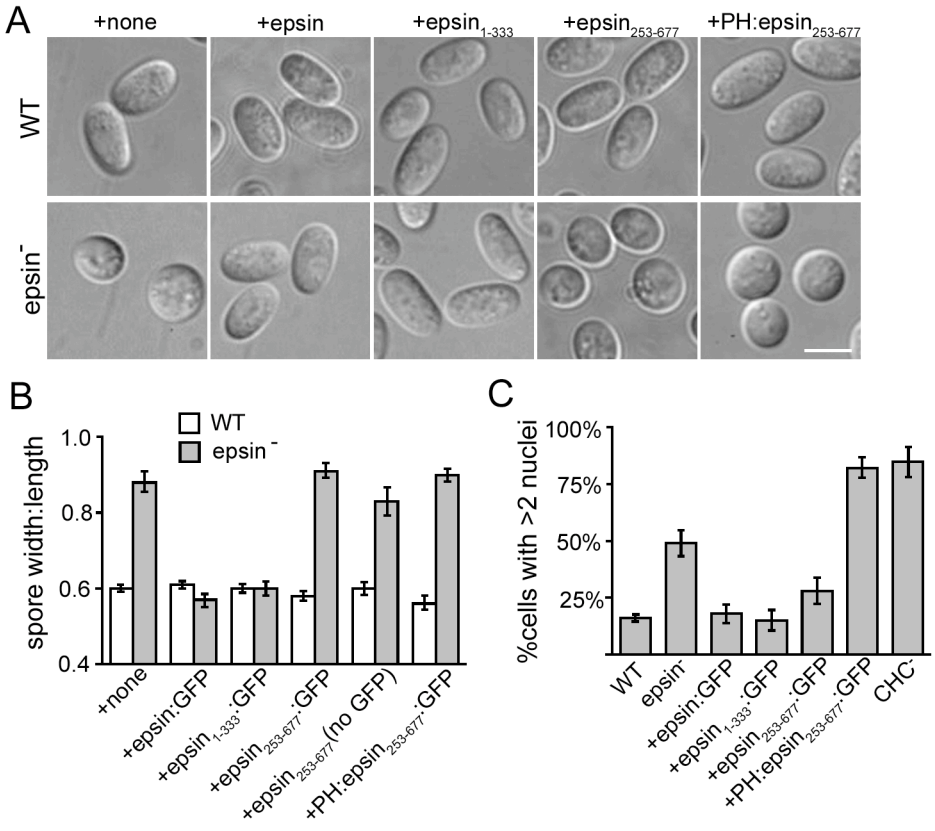

Figure 2.6. The ENTH domain rescues phenotypic defects of epsin mutants.

(A) DIC images of spores from wild-type (WT) and epsin mutants (epsin$^-$) expressing epsin:GFP, epsin$_{1\text{-}333}$:GFP, epsin$_{253\text{-}677}$:GFP, and PH:epsin$_{253\text{-}677}$:GFP. Bar, 5μm. (B) Ratio of spore length:width in wild-type (WT) and epsin null mutants (epsin-) and epsin null mutants expressing epsin:GFP, epsin$_{1\text{-}333}$:GFP, epsin$_{253\text{-}677}$:GFP, and PH:epsin$_{253\text{-}677}$:GFP; n = 50 for each cell line, error bars are standard error. (C) Quantification of multinucleated cells in suspension cultures of wild-type (WT), epsin mutants (epsin$^-$), clathrin mutants (clathrin-), and epsin mutants expressing epsin:GFP, epsin$_{1\text{-}333}$:GFP, epsin$_{253\text{-}677}$:GFP, and PH:epsin$_{253\text{-}677}$:GFP; n=300 for each cell line. Error bars are standard error.

We generated a construct that tagged the PLCδPH domain with GFP and examined its distribution in epsin null and wild-type cells. Consistent with membrane-binding properties similar to the ENTH domain of epsin, PLCδPH:GFP displayed a uniform plasma membrane localization comparable to $epsin_{1-333}$:GFP and did not disrupt clathrin localization or function (Appendix D, Figure D.6).

To determine whether this canonical $PI(4,5)P_2$ -binding domain could substitute for the ENTH domain function, we made a chimeric GFP-tagged epsin that replaced the ENTH domain with the PH domain of PLCδ (Figure 2.4). If this alternate $PI(4,5)P_2$ -binding domain was able to substitute for the ENTH domain, the PH-epsin C-terminal domain chimera (PH:$epsin_{253-677}$:GFP) should distribute similarly to full-length epsin. However, when expressed in wild-type cells, the PH:$epsin_{253-677}$:GFP chimera localized in a distinct and aberrant pattern. Instead of forming puncta evenly distributed on the plasma membrane and puncta within the cytoplasm, PH:$epsin_{253-677}$:GFP aggregated into large patches on the plasma membrane (Figure 2.7, A and B, compare Figure 2.5, A and B). Moreover, these aberrant patches sequestered both AP2 and clathrin. Staining with anti-AP2 antibody revealed that AP2 puncta frequently clustered within the PH:$epsin_{253-677}$:GFP patches (Figure 2.7A). Staining with anti-clathrin antibody revealed that PH:$epsin_{253-677}$:GFP caused severe mislocalization of clathrin. In wild-type cells or epsin null cells rescued with epsin:GFP, clathrin normally forms discrete puncta on the plasma membrane and in the perinuclear region of the cytoplasm (Figure 2.5B). However, in wild-type cells expressing PH:$epsin_{253-677}$:GFP, clathrin aggregated together with PH:$epsin_{253-677}$:GFP caps at the plasma membrane, and nearly all cytoplasmic and perinuclear clathrin staining was absent (Figure 2.7B). Epsin null cells expressing PH:$epsin_{253-677}$:GFP showed an identical distribution and mislocalization of AP2 and clathrin (Appendix

D, Figure D.8). Thus, substitution of an alternate PH domain for the ENTH domain allowed the chimeric epsin to associate with the plasma membrane, and allowed the C-terminal domain to bind clathrin and AP2. However, the chimeric epsin also sequestered AP2 and clathrin into abnormal patches on the plasma membrane.

Imaging living cells expressing the chimeric epsin revealed that its dynamic association with the plasma membrane was also aberrant (Figure 2.7C). Puncta of GFP-epsin associated transiently with the plasma membrane and could be seen to build up into a discrete spot that subsequently disappeared. In contrast, the patches of PH:epsin$_{253\text{-}677}$:GFP were static on the membrane and did not appear to form or disassemble. Both the PH:epsin$_{253\text{-}677}$:GFP chimera and epsin GFP were expressed in similar amounts (Appendix D, Figure D.9). We therefore concluded that substitution of the PH domain for the ENTH domain disrupted the capacity of epsin to form transient puncta on the plasma membrane.

2.2.8 Expression of PH:epsin$_{253\text{-}677}$:GFP impairs clathrin function

To determine whether the sequestration of clathrin on the membrane by PH:epsin$_{253\text{-}677}$ ablated clathrin function, we tested wild-type cells expressing PH:epsin$_{253\text{-}677}$:GFP for phenotypes typical of clathrin mutants: defective osmoregulation, cytokinesis failure, and abnormal development.

Clathrin mutants display defects in the size and activity of the contractile vacuole, an osmoregulatory organelle in *Dictyostelium,* and are therefore osmosensitive (O'Halloran and Anderson, 1992; Wang *et al*., 2003). To test whether expressing PH:epsin$_{253\text{-}677}$:GFP induced osmosensitivity, we shifted cells from media to water, and examined the contractile vacuole under Differential Interference Contrast (DIC) microscopy. Wild-type cells displayed an increase in contractile

vacuole activity, with the contractile vacuole swelling and discharging. Similar to clathrin light-chain mutants (Wang *et al.*, 2003), wild-type cells expressing PH:epsin$_{253-677}$:GFP developed abnormally large contractile vacuoles with prolonged cycles of expansion (Figure 2.7D). This effect was due to the expression of PH:epsin$_{253-677}$:GFP as the contractile vacuole activity was not altered in wild-type cells expressing full-length epsin, either of the two epsin truncations, or the PH domain alone (Appendix D, Figure D.10).

Clathrin is also critical for cytokinesis. Both clathrin light chain and clathrin heavy chain mutants are unable to divide in suspension cultures and become large and multinucleated (Niswonger and O'Halloran, 1997a; Wang *et al.*, 2003). When grown in suspension cultures, wild-type cells expressing PH:epsin$_{253-677}$:GFP also accumulated many nuclei to the same extent as clathrin heavy chain mutant cells, demonstrating a similarly severe defect in cytokinesis (Figure 2.6C and 2.7E). These defects showed that coupling the carboxy-terminus of epsin to an alternate membrane-binding domain induces dominant negative phenotypes in growing cells that are characteristic of clathrin mutants.

In contrast with the other clathrin deficiencies induced by expressing PH:epsin$_{253-677}$:GFP in wild-type cells, expression of PH:epsin$_{253-677}$:GFP did not impair wild-type cells during development. Clathrin mutants are not able to complete development and aggregate to form stunted structures(Niswonger and O'Halloran, 1997b; Wang *et al.*, 2003). However, wild-type cells expressing PH:epsin$_{253-677}$:GFP developed into fruiting bodies with a stalk and a sorus that appeared normal in structure (Appendix D, Figure D.11). Moreover, the fruiting bodies of wild-type cells expressing PH:epsin$_{253-677}$:GFP contained oblong spores identical in shape to wild-

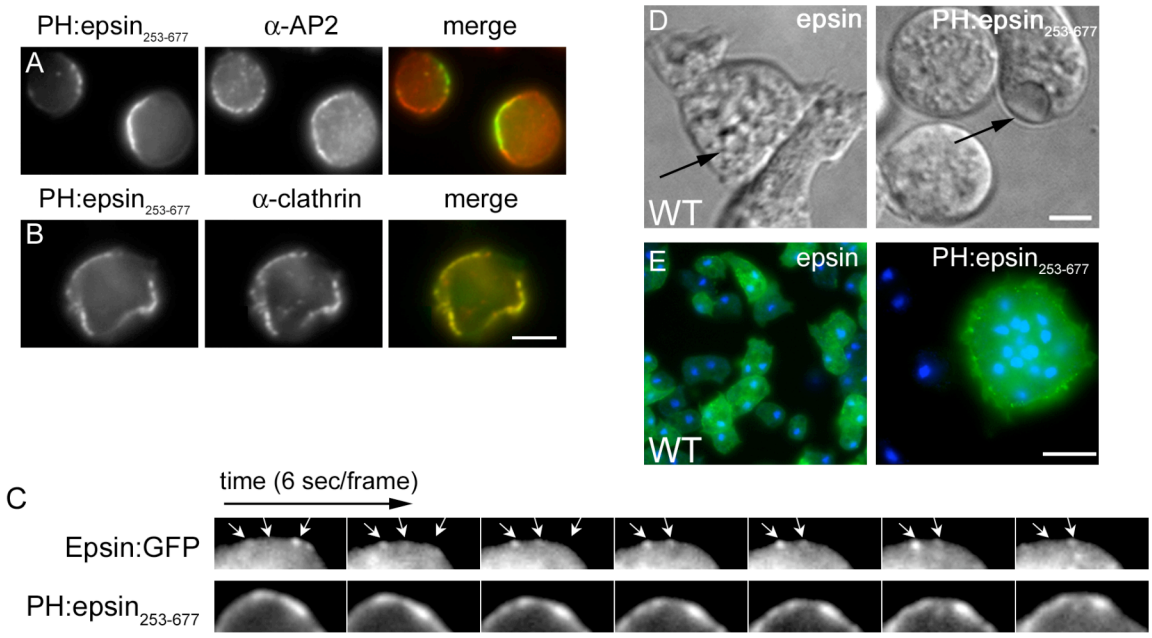

Figure 2.7. PLCδPH cannot functionally replace the ENTH domain.

(A and B) PH:epsin$_{253-677}$:GFP forms large patches on the plasma membrane, and cells expressing PH:epsin$_{253-677}$:GFP mislocalize clathrin and AP2 to these patches. Wild-type cells expressing PH:epsin$_{253-677}$:GFP (green) were fixed and immunostained with anti-alpha-adaptin antibody (A) or anti-clathrin antibody (B) (red). Bar, 5μm. (C) Epsin forms dynamic membrane puncta, but PH:epsin$_{253-677}$:GFP forms static patches. Plasma membrane images from a time course of wild-type cells expressing either epsin:GFP (top row) or PH:epsin$_{253-677}$:GFP (bottom row) were imaged under fluorescence miscroscopy (D and E) Wild-type cells expressing PH:epsin$_{253-677}$:GFP display clathrin-associated phenotypic defects, including enlarged contractile vacuoles and cytokinesis defects. (D) Wild-type cells expressing epsin:GFP or PH:epsin$_{253-677}$:GFP were shifted from media to water and imaged under DIC optics. Arrows, contractile vacuoles. Bar, 5μm. (E) Wild-type cells expressing epsin:GFP or PH:epsin$_{253-677}$:GFP (green) were cultured in suspension for three days, then fixed and stained with DAPI to visualize nuclei (blue). Bar, 10μm.

type spores, indicating that PH:epsin$_{253-677}$:GFP did not induce the formation of abnormal spores (Figure 2.6, A and B). Nonetheless, while the chimeric PH:epsin$_{253-677}$:GFP protein did not lead to dominant negative developmental phenotypes, the chimeric epsin also did not rescue the spore defect of epsin null cells. Examination of the spores housed within the sori of epsin null cells expressing the chimeric PH:epsin$_{253-677}$:GFP revealed round spores that were identical in morphology to the spores of epsin mutants (Figure 2.6, A and B).

2.2.9 Identification of residues in the ENTH domain important for localization and function

The inability of the PH domain to substitute for the ENTH domain suggested that the ENTH domain contributed more than PI(4,5)P_2-binding activity to epsin. To determine how the ENTH domain contributed to epsin localization and function, we first asked whether the PI(4,5)P_2-binding ability of the ENTH domain was critical for epsin function. Amino acids R65 and K78 have been shown to be critical for the interaction between the ENTH domain and PI(4,5)P_2 (Itoh *et al.*, 2001). To directly test the importance of this activity, we constructed two plasmids to express mutant versions of either the ENTH domain or full-length epsin with the R65A/K78A mutations. Assessment of the PI(4,5)P_2-binding capacity of ENTH$^{R65A/K78A}$ confirmed that mutating these residues impaired the ability of the ENTH domain to bind PI(4,5)P_2. (Figure 2.8A). Examination of the cells expressing the GFP-tagged proteins revealed that ENTH$^{R65A/K78A}$ and epsin$^{R65A/K78A}$ failed to associate with the plasma membrane (Figure 2.8, B and C), consistent with the insufficiency of the C-terminal domain to associate with clathrin-coated pits. Moreover, ENTH$^{R65A/K78A}$ and epsin$^{R65A/K78A}$ also failed to rescue the round spore phenotype, demonstrating that the

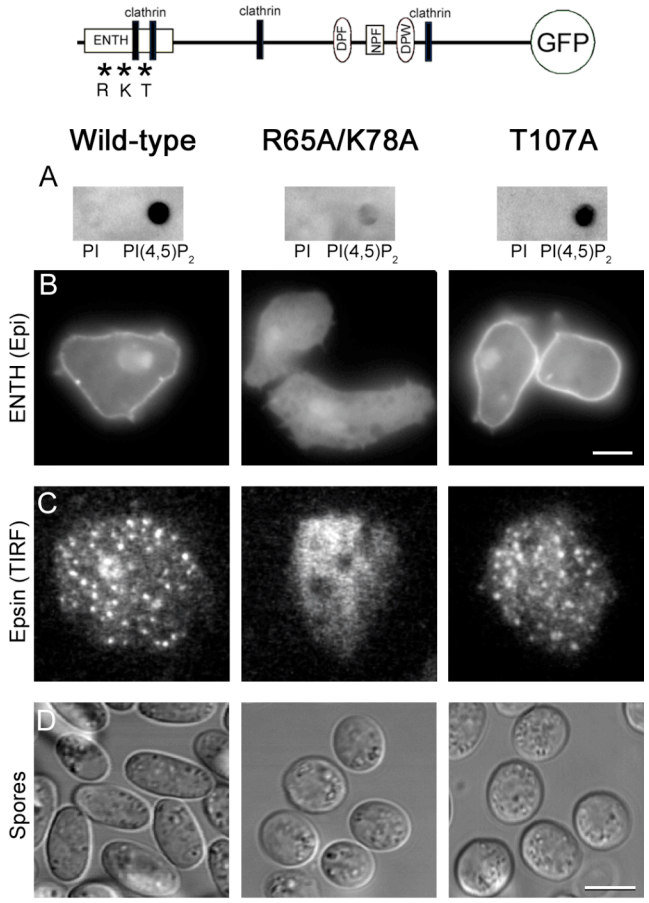

Figure 2.8. Epsin^{T107A} localizes to plasma membrane puncta but does not rescue spore morphology.

(A) ENTH$^{R65A/K78A}$, but not ENTH^{T107A}, has impaired binding to PI(4,5)P_2. PI and PI(4,5)P_2 were pipetted onto nitrocellulose membrane and then incubated with lysate from *Dictyostelium* cells expressing either wild-type or mutant ENTH:GFP. Blots were probed with anti-GFP antibody. (B) Wild-type or mutant ENTH:GFP was expressed in epsin null cells and imaged by epifluorescence widefield microscopy (epi). ENTH^{WT} (left) and ENTH^{T107A} (right) localize to the plasma membrane, while ENTH$^{R65A/K78A}$ (center) does not. (C) Wild-type or mutant epsin:GFP was expressed in epsin null cells and imaged by total internal reflection microscopy (TIRF). Epsin^{T107A} (right) forms puncta similar to epsin^{WT} (left). Epsin$^{R65A/K78A}$ (center) does not form puncta on the plasma membrane. (D) Epsin null cells expressing wild-type or mutant ENTH:GFP were allowed to develop on starvation plates. Spores were harvested and imaged under DIC optics. ENTH$^{R65A/K78A}$ (center) or ENTH^{T107A} (right) can not rescue spore morphology.

ability to bind $PI(4,5)P_2$ was required for both epsin function and localization (Figure 2.8D). We also tested the contribution of another amino acid within the ENTH domain, T107, a residue not predicted to function in $PI(4,5)P_2$ binding. The analogous amino acid in yeast epsin is essential for viability (Aguilar *et al*., 2006) demonstrating an important contribution to epsin activity. The contribution of this residue to epsin localization has not been examined. To test the contribution of T107 to *Dictyostelium* epsin, we constructed a plasmid that introduced the T107A mutation into the ENTH domain and full-length epsin. In contrast with epsin$^{R65A/K78A}$, the T107A mutation did not affect the localization of epsin. ENTH^{T107A} was distributed uniformly along the plasma membrane, and epsin^{T107A} localized within puncta on the plasma membrane (Figure 2.8, B and C). Similarly to wild-type epsin, these puncta colocalized with plasma membrane clathrin (see Chapter 3). However, despite the wild-type association with the plasma membrane or coated pits, neither ENTH^{T107A} nor epsin^{T107A} were able to rescue the round-spore phenotype of epsin null cells (Figure 2.8D). Thus this mutation separates the contribution of the ENTH domain to epsin localization from its contribution to essential cellular function.

2.3 DISCUSSION

Epsin is a phylogenetically conserved clathrin adaptor protein. Our results define determinants essential for targeting epsin into clathrin-coated pits that are distinct from determinants essential for epsin function. In this work we identified clathrin, but not AP2, as essential for epsin localization within clathrin-coated pits. Our analysis also demonstrated that a cooperative interaction between the two domains of epsin enables this protein to interact dynamically with clathrin pits on the plasma membrane. Our results support a model where the ENTH domain coordinates

with the clathrin-binding C-terminal domain to tether epsin to the plasma membrane for a productive and functional interaction with clathrin-coated pits. Independent of targeting to coated pits, the isolated ENTH domain is both necessary and sufficient for rescuing tthe aberrant spore morphology of epsin null cells. Thus, determinants for targeting epsin to coated pits are distinct from those that supply function.

2.3.1 Clathrin, but not AP2, is a determinant for localizing epsin within coated pits

By examining the localization of epsin in different mutant backgrounds, we were able to define how other proteins contribute to the localization of epsin within clathrin-coated pits. We identified clathrin heavy chain as necessary for epsin to cluster to plasma membrane puncta, as clathrin heavy chain nulls distributed epsin uniformly on the plasma membrane and contained more soluble epsin than wild-type cells. Among *Dictyostelium* clathrin-associated proteins, this requirement for clathrin to form puncta is unique, as clathrin mutants continue to exhibit both AP2 and AP180 continue to form puncta on the membrane of clathrin mutants (Stavrou and O'Halloran, 2006; Wen and O'Halloran, unpublished results).

By contrast, epsin continued to cluster within clathrin-coated pits in AP2-alpha mutants. Consistent with depletion experiments in vertebrate cell culture, (Hinrichsen *et al.*, 2003; Motley *et al.*, 2003), deletion of AP2 in *Dictyostelium* caused a marked decrease in the total number of puncta at the membrane that contain epsin and clathrin. Although the number of epsin puncta is reduced in AP2-alpha mutants, the remaining epsin puncta continue to colocalize with clathrin. Thus, while AP2 increases the number of clathrin puncta on the membrane, the interaction between AP2 and epsin is not critical for epsin to incorporate into clathrin-coated pits.

Similarly, we have found that deletion of other *Dictyostelium* clathrin accessory proteins, including Hip1r and AP180, does not affect epsin localization to clathrin-coated pits (Stavrou and O'Halloran, 2006; Repass, *et al*. 2006).

We found that *Dictyostelium* epsin null cells manifested only limited clathrin-associated phenotypes. In epsin null cells, epsin function may be covered by alternate clathrin adaptors, including AP2 and AP180. Similarly to epsin, AP2 and AP180 bind $PI(4,5)P_2$ and contain multiple motifs for interacting with other coated pit proteins (Edeling et al., 2006; Legendre-Guillemin et al., 2004).Our findings in epsin null mutants may indicate that epsin is not important for initiating clathrin pit assembly, and are consistent with a more specialized role for epsin in recruiting and processing specific cargo rather than assembly of the clathrin lattice itself (Wang et al., 2006a; Wang and Struhl, 2005).

2.3.2 Contribution of the carboxy-terminal domain

In addition to examining how other proteins contribute to epsin localization, we also defined determinants within the epsin protein necessary and sufficient targeting within coated pits. As with other epsins, the *Dictyostelium* epsin C-terminal domain contained motifs for interacting with coated pits. These motifs included clathrin and AP2 binding motifs, but not a motif for interacting with ubiquitin. The latter motif is also lacking in *Arabidopsis* epsin (Holstein and Oliviusson, 2005). Surprisingly, we found that, while essential, the clathrin-binding C-terminal domain was not sufficient for associating with clathrin pits. Coupling the C-terminal domain to an alternate membrane-binding domain, a PH domain, created a chimeric molecule capable of associating with clathrin and AP2 on the plasma membrane. Neither the C-terminal domain nor the PH domain on its own associated with clathrin on the

membrane, confirming the potent capacity of the C-terminal domain to associate with clathrin pits, but highlighting the additional requirement for a membrane-targeting domain. However, the chimeric PH:epsin$_{253-677}$ molecule was not functional, and even sequestered clathrin to the extent of abolishing clathrin function. Thus the C-terminal domain is necessary but not sufficient for the functional interaction of epsin with clathrin-coated pits; the ENTH domain is also required. Moreover, the nonproductive and static interaction of the chimeric molecule with clathrin at the plasma membrane suggests that the ENTH domain tempers the clathrin-binding ability of the C-terminal domain, allowing the interaction between epsin and clathrin to be transient, dynamic, and functional.

2.3.3 The ENTH domain: essential for localization; sufficient for function

The insufficiencies of the isolated C-terminal domain highlight a unique contribution of the ENTH domain to both the localization and function of epsin. The ENTH domain binds to PI(4,5)P_2 on the plasma membrane, a phospholipid critical for coated pit assembly (Zoncu *et al.*, 2007). The C-terminal domain of epsin required this membrane-binding function of the ENTH domain in order to cluster within clathrin pits. However, the disruption of clathrin distribution and dominant negative phenotypes associated with expression of the PH:epsin$_{253-677}$ chimera suggested a new function for the ENTH domain in modulating the clathrin binding capacity of epsin.

Although the ENTH domain is essential for epsin localization, it is not sufficient. The ENTH domain alone could not cluster to clathrin-coated pits, but instead distributed uniformly over the plasma membrane. By contrast, the ENTH domain was both necessary and sufficient to rescue the spore morphology defects of epsin null mutants. Thus, the determinants sufficient for clustering epsin within

clathrin-coated pits, which require both the C-terminal domain and the ENTH domain, are distinct from the determinants sufficient for supplying function, which are contained solely in the ENTH domain.

2.3.4 Determinants within the ENTH domain that contribute to epsin function

Mutating residues R65 and K78 in the isolated ENTH domain ablated $PI(4,5)P_2$ binding and also ablated the capacity of the ENTH domain to rescue epsin null phenotypes. Similarly, mutating R65/K78 in full-length epsin also rendered the protein unable to bind to coated pits and compromised its ability to rescue the round spore phenotype of epsin mutants. This mutant demonstrates that the ability of the ENTH domain to bind $PI(4,5)P_2$ is required for both epsin localization and essential function. In contrast, the mutating the T107 residue rendered epsin non-functional, but still able to localize within clathrin-coated pits. Thus this residue within the ENTH domain contributes to the essential function of epsin, but does not contribute to the ability of epsin to target to and incorporate within clathrin-coated pits.

How does the T107 residue contribute to epsin function? The analogous residue in the yeast epsin homolog is part of a functional patch that binds to a GTPase activating protein (GAP) for cdc42 and contributes to regulation of the actin cytoskeleton and cell polarity (Aguilar *et al*., 2006). The mechanism by which the T107 residue contributes to epsin function may be different than in yeast, as the *Dictyostelium* genome does not contain a gene for cdc42. However, the *Dictyostelium* ENTH domain still may be supplying a similar function by determining the polar organization of cellular components in the oblong spore. At present, little is known about how *Dictyostelium* spores construct their oblong shape. Interestingly, the clathrin accessory protein Hip1r also forms abnormally round spores in *Dictyostelium*

(Repass *et al.*, 2007). Moreover, the ENTH domain of epsin is required for the phosphorylation and coated pit localization of Hip1r. An important function of the ENTH domain may be to regulate the localization and activity of Hip1r.

2.3.5 Functional contribution of the ENTH domain

The ability of the isolated ENTH domain to rescue epsin null phenotypes even though it does not localize within coated pits suggests that epsin could have two distinct functions. One is an essential developmental function that contributes to spore morphology. Epsin does not require coated-pit localization in order to operate in this capacity, suggesting that this activity may be independent of clathrin. The other function of epsin is within clathrin-coated pits on the plasma membrane.

What is the functional contribution of epsin to clathrin-coated pits? *In vitro* studies demonstrate that epsin promotes invagination of clathrin assembled on lipid monolayers (Ford *et al.*, 2002). More recently, *in vivo* studies have suggested that clathrin itself is the driving force in coated pit invagination from the plasma membrane (Hinrichsen *et al.*, 2006). Consistent with this observation, we found that *Dictyostelium* epsin null cells manifested only limited clathrin-associated deficits. Our results argue for a more specialized role for epsin during clathrin-mediated endocytosis. It has been suggested that adaptors such as epsin are involved in sorting ligands to distinct endosomal populations (Lakadamyali *et al.*, 2006) and allow precise endocytosis of certain surface receptors critical for appropriate cell fate specification (Berdnik *et al.*, 2002; Overstreet *et al.*, 2003; Traub, 2003; Wang and Struhl, 2005). Dissecting how epsin and other adaptors function in eukaryotic cells to tailor clathrin-coated pits amidst large volumes of membrane traffic remains an important challenge.

Chapter 3: Epsin ENTH domain regulates actin dynamics during clathrin-mediated endocytosis through Hip1r

3.1 INTRODUCTION

Clathrin-mediated endocytosis is an essential cellular process involved in nutrient uptake, processing of extracellular signals, and membrane remodeling. In clathrin-mediated endocytosis, clathrin forms a coated pit around transmembrane cargo on the plasma membrane in association with several accessory factors. These coated pits bud off into the cell, creating small clathrin-coated vesicles (Brodsky et al., 2001). Adaptor proteins help to organize the clathrin-coated pit and link clathrin to endocytic cargo and the plasma membrane. Several adaptors have been identified and their interaction with clathrin well characterized (Owen et al., 2004; Robinson, 2004).

Epsin is a clathrin adaptor with a modular organization. At its amino-terminus, epsin contains an ENTH (*e*psin *N*-*t*erminal *h*omology) domain that interacts with the membrane by binding $PI(4,5)P_2$ (Itoh et al., 2001). The ENTH domain inserts into the membrane when bound to $PI(4,5)P_2$, inducing curvature (Ford et al., 2002). C-terminal to the ENTH domain are a series of short motifs that bind specifically to clathrin, AP2, and EH domain containing proteins (Chen et al., 1998; Drake et al., 2000; Owen et al., 1999; Traub et al., 1999). These two halves of epsin cooperate to facilitate a dynamic interaction with clathrin-coated pits on the plasma membrane (see Chapter 2).

The clathrin adaptor Hip1r contains an amino-terminal ANTH (*A*P180 *N*-*t*erminal *h*omology) domain which binds to $PI(4,5)P_2$ but does not induce curvature (Ford et al., 2002; Sun et al., 2005). Hip1r interacts with clathrin by binding to

clathrin light chain via a central coiled-coil domain, which also mediates homodimerization (Engqvist-Goldstein et al., 2001; Legendre-Guillemin et al., 2005; Wesp et al., 1997). The C-terminus of Hip1r contains a THATCH (*t*alin-*H*IP1/R/Sla2p *a*ctin-*t*ethering *C*-*t*erminal *h*omology) domain, also known as an I/LWEQ domain. The THATCH domain binds to F-actin, suggesting that Hip1r functions as a linker between clathrin and the actin cytoskeleton (Engqvist-Goldstein et al., 1999).

An interaction between epsin and Hip1r was first suggested by the finding that truncating the THATCH domain of Sla2p, the yeast Hip1r homolog, caused synthetic temperature sensitive endocytic defects in an *ent2Δ* mutant, the yeast epsin homolog (Baggett et al., 2003). A more direct functional link between epsin and Hip1r has recently been described in *Dictyostelium*, where epsin is required for the localization and phosphorylation of Hip1r (Repass et al., 2007). How epsin regulates Hip1r to promote its interaction with dynamic, functional clathrin-coated pits at the plasma membrane remains unclear.

Here, we provide evidence that both epsin and Hip1r regulate the dynamic interaction of actin with clathrin-coated pits just before internalization in *Dictyostelium*. We have identified residues in the ENTH domain of epsin that are critical for this function. We propose that the ENTH domain of epsin facilitates the membrane recruitment and phosphorylation of Hip1r, which in turn mediates the productive interaction of clathrin with the actin cytoskeleton at the plasma membrane.

3.2 RESULTS

3.2.1 Hip1r localizes to plasma membrane clathrin puncta that contain epsin

Both epsin and Hip1r associate with clathrin puncta on the plasma membrane of *Dictyostelium* cells (Repass, et al., 2007, see Chapter 2). To determine the extent of simultaneous colocalization between Hip1r, epsin and clathrin, wild-type cells co-expressing epsinGFP and clathrinRFP were fixed and immunostained with anti-Hip1r antibodies. When examined under epifluorescence microscopy, epsin, clathrin, and Hip1r formed discrete puncta on the plasma membrane (Figure 3.1A). Quantification of these puncta revealed that 50% ± 3% (mean ± SE, n = 15 cells) of Hip1r puncta colocalized with puncta containing both epsin and clathrin, while 22% ± 3% of Hip1r puncta colocalized with puncta containing epsin without clathrin. Only 4% ± 1% of Hip1r puncta colocalized with clathrin but not with epsin (Figure 3.1C). Thus, Hip1r predominantly localizes to clathrin and epsin positive puncta, and rarely associates with clathrin in the absence of epsin.

To determine if the association of Hip1r with clathrin and epsin puncta was unique to Hip1r, we assessed the level of colocalization between clathrin, epsin, and another adaptor, AP2. AP2 is the predominant and best-characterized clathrin adaptor at the plasma membrane. Wild-type cells co-expressing epsinGFP and clathrinRFP were immunostained with antibodies against the α-adaptin subunit of AP2 and imaged under epifluorescence microscopy. Similar to clathrin, Hip1r, and epsin, AP2 formed discrete puncta on the plasma membrane (Figure 3.1B). 49% ± 4% (mean ± SE, n = 12 cells) of AP2 puncta also contained both clathrin and epsin, and 20% ± 4% of AP2 puncta contained epsin with out clathrin. Only a small amount of AP2 puncta, 8% ± 2%, associated with clathrin in the absence of epsin (Figure 3.1D). The amount

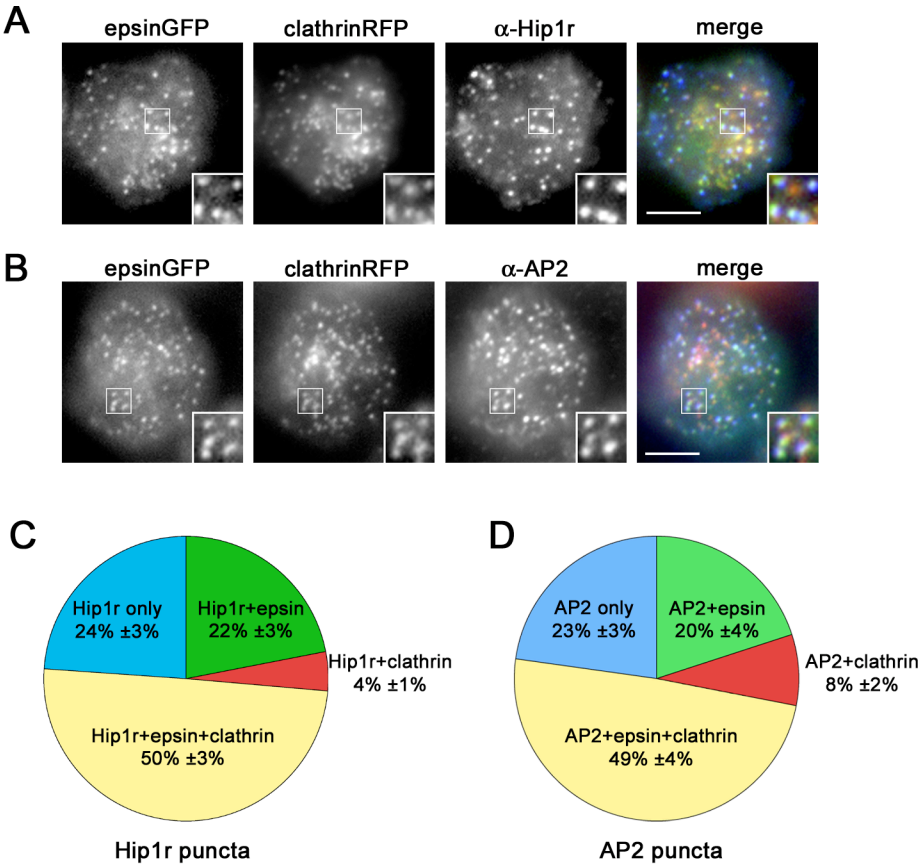

Figure 3.1. Hip1r associates with epsin and clathrin puncta in similar proportions as AP2

(A) Wild-type cells co-expressing epsinGFP and clathrinRFP were fixed and immunostained with anti-Hip1r antibodies. Cell surfaces were imaged under epifluorescence microscopy. Bar, 5μm. (B) Wild-type cells co-expressing epsinGFP and clathrinRFP were fixed and immunostained with anti-AP2 antibodies and imaged as in (A). Bar, 5μm. (C) Proportion of Hip1r puncta that colocalized with epsin, clathrin, or epsin and clathrin simultaneously. (D) Proportion of AP2 puncta that colocalized with epsin, clathrin, or epsin and clathrin simultaneously.

of association of AP2 puncta with clathrin and epsin at the membrane was similar to that of Hip1r puncta, suggesting that this distribution is typical of clathrin adaptors.

3.2.2 Blocking actin polymerization increases association of Hip1r with clathrin-coated pits.

Actin plays an important role in late stages of clathrin-mediated endocytosis. In yeast and mammalian cell culture, treatment with actin depolymerizing drugs arrests clathrin pits on the plasma membrane (Merrifield *et al*., 2005; Newpher *et al*., 2005). To determine if actin is important for clathrin dynamics in *Dictyostelium*, we treated wild-type cells expressing clathrinRFP with the actin depolymerizing drug cytochalasin A. Clathrin normally forms puncta at the plasma membrane and in the cytoplasm, and strongly labels the perinuclear region of the cell (Figure 3.2A, left panel). After one hour of 20μM cytochalasin A treatment, clathrin puncta accumulated at the plasma membrane with fewer clathrin puncta in the cytoplasm (Figure 3.2A, right panel), suggesting that actin is required for late stages of clathrin pit maturation in *Dictyostelium*.

Hip1r, which binds to actin via its THATCH domain, has been proposed to link clathrin pits to the actin cytoskeleton (Engqvist-Goldstein et al., 2001). To determine if the association of Hip1r with clathrin-coated pits is sensitive to perturbations in the actin cytoskeleton, we treated wild-type cells expressing epsinGFP and clathrinRFP with cytochalasin A, then fixed and immunostained for either Hip1r or AP2 (Figure 3.2, B and C). The association of AP2 with clathrin and epsin positive puncta increased only slightly with cytochalasin A treatment, from 49% to 57%, ± 3%. By contrast, the percentage of Hip1r that associated with both epsin and clathrin increased from 50% in untreated cells to 72% ± 5% in treated cells

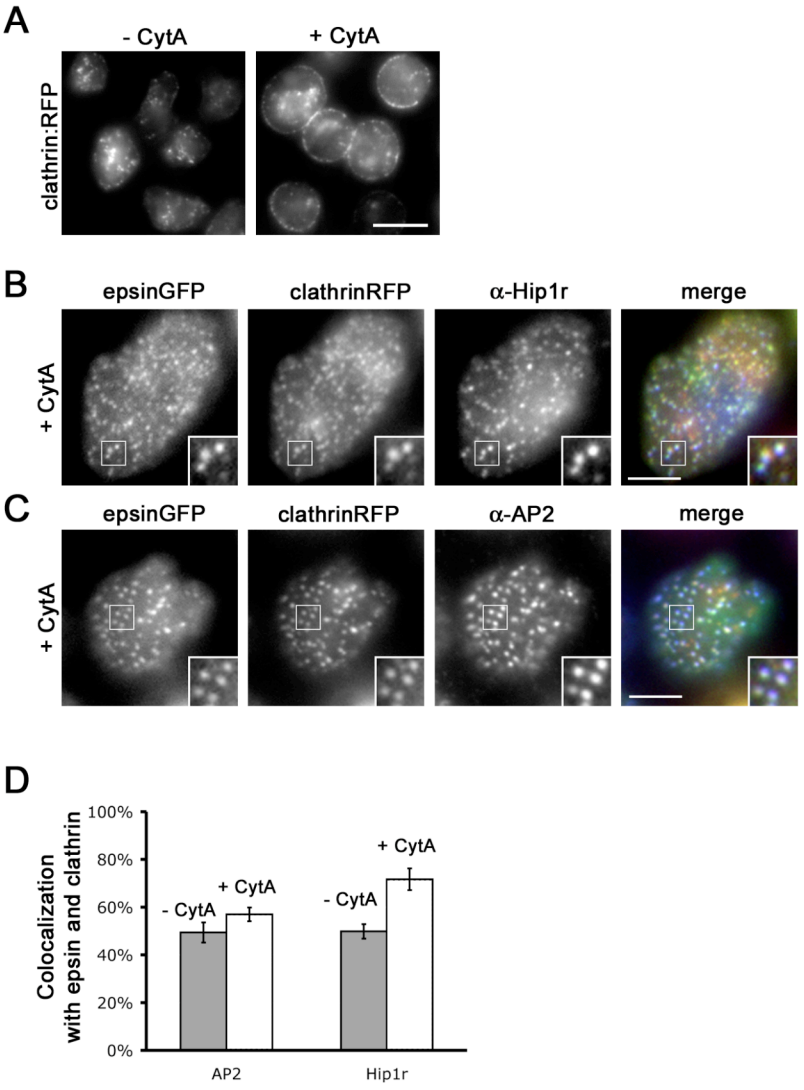

Figure 3.2. Treatment with cytochalasin A increases the association of Hip1r with clathrin-coated pits.

(A) Treatment with cytochalasin A leads to an accumulation of clathrin at the membrane. Wild-type cells expressing clathrinRFP were treated with 20μM cytochalasin A for 1 hr then imaged under epifluorescence microscopy. Bar, 10μm. (B and C) Wild-type cells co-expressing epsinGFP and clathrinRFP were treated with cytochalasin A as in (A), fixed, and immunostained with either anti-Hip1r (B) or anti-AP2 (C) antibodies. Bar, 5μm. (D) Quantification of colocalization between epsin, clathrin, and either Hip1r or AP2 in the presence or absence of cytochalasin A treatment. Error bars are standard error.

(Figure 3.2D). Thus, inhibiting dynamic actin stabilized the association of Hip1r with clathrin-coated pits on the plasma membrane.

3.2.3 Inhibiting dynamic actin stabilizes clathrin-associated Hip1r puncta on the plasma membrane of epsin null cells

We have previously shown that Hip1r requires epsin to localize to clathrin pits on the plasma membrane (Repass et al., 2007). To determine if the requirement for epsin in Hip1r localization is sensitive to actin dynamics, we treated epsin-null cells with cytochalasin A and immunostained for Hip1r (Figure 3.3). In untreated wild-type cells, Hip1r formed discrete puncta on the plasma membrane. When wild-type cells were treated with cytochalasin A, the number and intensity of Hip1r puncta on the membrane increased (Figure 3.3A, top row). In untreated epsin-null cells, Hip1r did not form membrane-associated puncta, but instead formed puncta in the cytoplasm. However, when epsin-null cells were treated with cytochalasin A, Hip1r formed puncta on the plasma similar to wild-type cells (Figure 3.3A, bottom row). Thus, treatment with Cytochalasin A abrogated the requirement for epsin in the formation of membrane-associated Hip1r puncta.

To determine whether the Hip1r puncta formed in epsin-null cells treated with cytochalasin A associated with clathrin, we treated both wild-type and epsin-null cells expressing clathrinGFP with cytochalasin A and immunostained for Hip1r. Examining these cells under epifluorescence microscopy revealed that Hip1r puncta at the membrane of cytochalasin A-treated epsin null cells colocalized with clathrin. Quantification of the colocalization of Hip1r with clathrin showed that in wild-type cells treated with cytochalasin A, 76% ± 5% of Hip1r puncta colocalized with clathrin on the plasma membrane. In epsin null cells, cytochalasin-induced Hip1r puncta

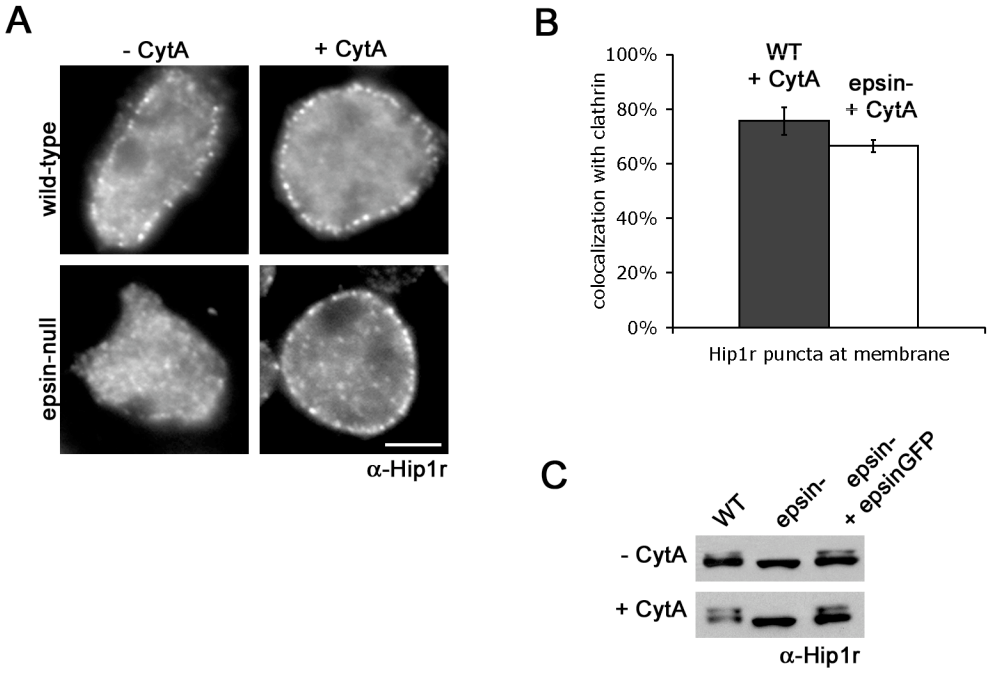

Figure 3.3. Cytochalasin A treatment can induce Hip1r puncta at the membrane of epsin null cells but cannot induce phosphorylation of Hip1r.

(A) Cytochalasin A treatment causes the formation of Hip1r puncta at the membrane. Wild-type and epsin null cells (**- CytA**, left column) were fixed and immunostained with anti-Hip1r antibodies. Wild-type and epsin null cells were treated with 20μM cytochalasin A (**+ CytA**, right column), fixed, and immunostained for Hip1r. Bar, 5μm. (B) Cytochalasin A-induced puncta colocalize with clathrin. Wild-type or epsin null cells expressing clathrinGFP were fixed treated with cytochalasin A, fixed, and immunostained for Hip1r. Cells were imaged under fluorescence microscopy and colocalization quantified. Error bars are standard error. (C) Cytochalasin A treatment cannot induce Hip1r phosphorylation. Cell lysates from wild-type (**WT**), epsin null (**epsin-**), and epsin null cells expressing epsinGFP (**epsin- + epsinGFP**) treated with cytochalasin A were analyzed by immunoblot and probed with anti-Hip1r antibodies.

colocalized with clathrin at slightly reduced levels of 67% ± 2% (Figure 3.3B). This indicates Hip1r does not require epsin to associate with clathrin. However, epsin is required for this association between Hip1r and clathrin to occur on the plasma membrane in the presence of dynamic actin.

3.2.4 Epsin is required for Hip1r phosphorylation

Hip1r is phosphorylated *in vivo*. This phosphorylation event requires epsin, as Hip1r is not phosphorylated in epsin null cells (Repass et al., 2007). Conceivably, inducing the formation of Hip1r puncta at the membrane of epsin null cells with cytochalasin A treatment may also induce Hip1r phosphorylation. To test this possibility, we treated wild-type and epsin null cells with cytochalasin A and analyzed the cell lysates by immunoblot using anti-Hip1r antibodies. As described previously (Repass et al., 2007), blots of lysates from untreated wild-type cells display two species of Hip1r, an unphosphorylated lower band and a phosphorylated upper band. Both species of Hip1r were also present in lysates of wild-type cells treated with cytochalasin A and epsin null cells expressing epsinGFP (Figure 3.3C). By contrast, lysates from epsin null cells only contained the lower, unphosphorylated form of Hip1r. Blots of lysates from epsin null cells treated with cytochalasin A also displayed only the lower, unphosphorylated form of Hip1r (Figure 3.3C), indicating that membrane localization alone is not sufficient for Hip1r phosphorylation; there is a strict requirement for epsin.

3.2.5 Epsin is required for the efficient internalization of clathrin coated pits at the plasma membrane

We have demonstrated that epsin affects the recruitment of a specific clathrin adaptor, Hip1r, to the plasma membrane. To determine if epsin also influences the dynamic assembly of clathrin into puncta on the plasma membrane, we examined wild-type and epsin null cells expressing clathrinRFP using total internal reflection fluorescence (TIRF) microscopy. To avoid UV-damage, we imaged cells for 120 seconds, a period in which the cells continued their normal behavior. In wild-type cells, clathrin formed discrete puncta that formed and then disappeared from the membrane (Figure 3.4). These puncta persisted on the membrane an average of 26 ± 3 sec (n= 10 puncta on 4 cells). 97% of clathrin puncta in wild-type cells identified at the beginning of a timelapse capture were internalized within 60 seconds, with no puncta persisting longer than 120 seconds (Figure 3.4, A and B). By contrast, clathrin puncta in epsin null cells persist at the membrane much longer (Figure 3.4A). Only 50% of clathrin puncta in epsin null cells were internalized within 60 seconds, and 23% of clathrin puncta persisted longer than 120 seconds (Figure 3.4B). The increased persistence of clathrin at the membrane in epsin null cells, which extended beyond the 120-second imaging period, prevented the calculation of an average lifetime in these mutants. This indicates that the absence of epsin interrupts internalization of clathrin-coated pits or a stage just prior to internalization.

3.2.6 Epsin null cells display abnormal actin dynamics at the plasma membrane

Actin is involved in late stages of clathrin-mediated endocytosis, including clathrin pit internalization. The defect in clathrin dynamics in epsin null cells may be a result of defective actin dynamics. To visualize the actin dynamics in epsin-null

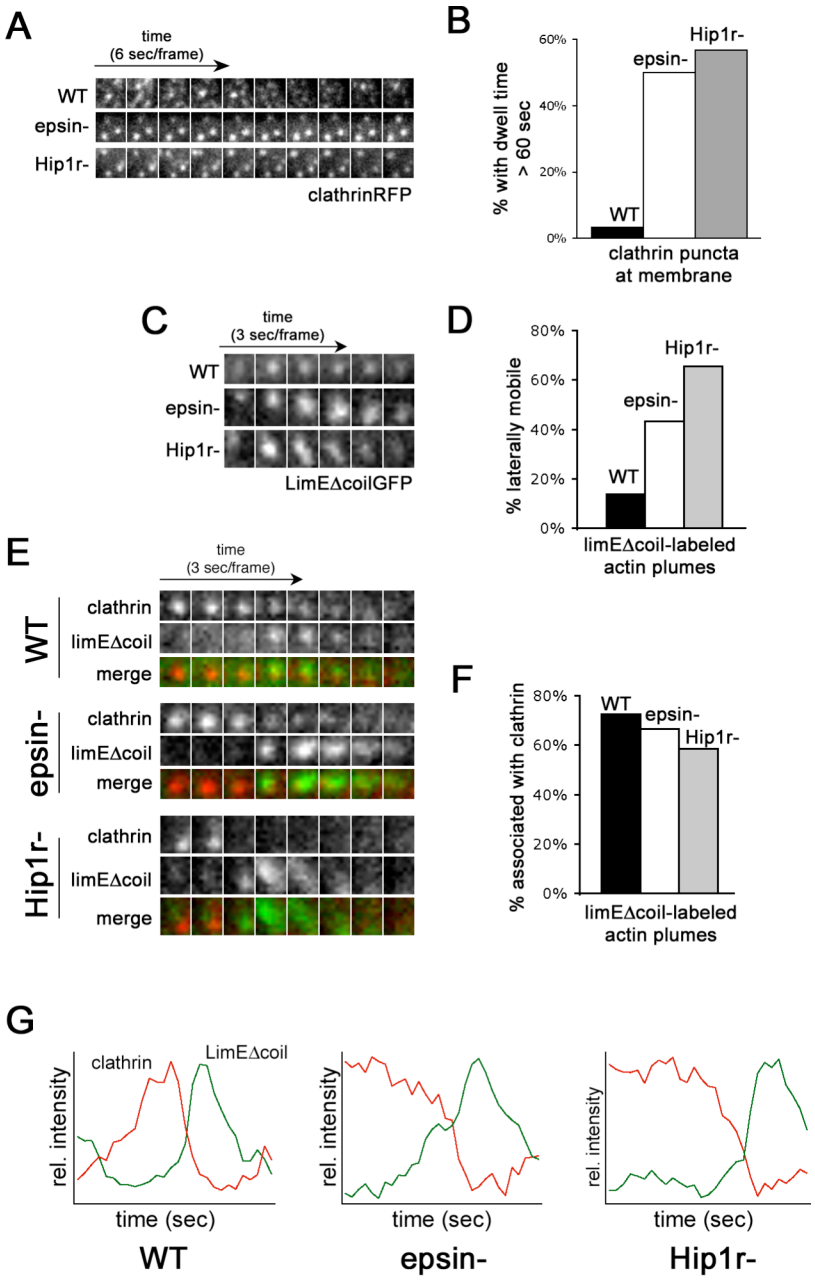

Figure 3.4: Epsin and Hip1r are necessary for wild-type actin polymerization at sites of clathrin internalization.

(A and B) Clathrin puncta persist at the membrane longer in epsin and Hip1r null mutants. (A) Time lapse montage of wild-type (**WT**), epsin null (**epsin-**), and Hip1r null (**Hip1r-**) cells expressing clathrinRFP and imaged under TIRF microscopy.

(Figure 3.4, continued) (B) Quantification of persistence of individual clathrin puncta on the surface of wild-type, epsin null, and Hip1r null cells. Puncta were identified at the beginning of imaging and tracked until they disappeared. n=30 puncta for each cell line (C and D) Transient actin puncta, as visualized by limEΔcoilGFP, display increased lateral mobility in epsin and Hip1r null mutants. (C) Timelapse montage of wild-type, epsin null, and Hip1r null cells expressing limEΔcoilGFP and examined under TIRF microscopy. (D) Quantification of lateral mobility individual limEΔcoil plumes on the surface of wild-type, epsin null, and Hip1r null cells. n=30 puncta for each cell line. (E and F) Actin puncta appear at sites of clathrin internalization in wild-type, epsin null, and Hip1r null mutants. (E) Timelapse montage of wild-type, epsin null, and Hip1r null cells co-expressing clathrinRFP and limEΔcoilGFP and examined under TIRF microscopy. (F) Quantification of association between individual limEΔcoil-labeled actin puncta and clathrin puncta on the surface of wild-type, epsin null, and Hip1r null cells. n=30 puncta for each cell line. (G) representative graphs of clathrin and limEΔcoil signal at a specific puncta in wild-type (**WT**), epsin null (**epsin-**) or Hip1r null (**Hip1r-**) cells.

cells, we expressed LimEΔcoilGFP, a construct that preferentially labels filamentous actin (Bretschneider et al., 2004). Wild-type cells expressing LimEΔcoilGFP and examined under TIRF microscopy displayed localized, transient bursts of actin polymerization lasting 15 ± 1 seconds (mean ± SE, n = 30 puncta). These actin bursts appeared as small, round puncta that displayed little lateral movement (Figure 3.4, C and D). Epsin null cells expressing LimEΔcoilGFP also displayed transient bursts of actin labeled with LimE that lasted slightly longer, 18 ± 1 seconds. However, these actin spots were more diffuse, slightly larger, irregularly shaped, and moved laterally on the membrane before disappearing (Figure 3.4C). 43% of transient actin puncta in epsin null cells (n = 30 puncta; 3 cells) moved laterally compared to only 14% of wild-type actin puncta (Figure 3.4D). This suggested that, directly or indirectly, epsin regulates both the morphology and the mobility of transient actin puncta on the plasma membrane.

3.2.7 Actin puncta associate with internalizing clathrin puncta on the membrane of wild-type *Dictyostelium* cells

It has been reported that short bursts of actin polymerization accompany the internalization of clathrin puncta from the membrane (Merrifield *et al.*, 2005; Newpher *et al.*, 2005). To visualize this process in *Dictyostelium*, we co-expressed clathrinRFP and LimEΔcoilGFP in wild-type cells and imaged these cells using TIRF microscopy. In wild-type cells, clathrin puncta increase in intensity before rapidly disappearing from the membrane, presumably by internalization. Frequently, an actin punctum, as visualized by LimEΔcoilGFP, accompanied the disappearance of these clathrin puncta (Figure 3.4). 48% of clathrin puncta identified at the beginning of timelapse image capture eventually associated with a transient actin puncta before internalization in wild-type cells. This indicates that actin could be important, but not universal, component of clathrin-coated pit internalization. Although a large portion of clathrin puncta disappeared without actin polymerization, 72% of transient actin puncta forming at the membrane in wild-type cells were associated with a clathrin-coated pit (Figure 3.4, E and F). All clathrin puncta that associated with an actin punctum disappeared from the membrane. This suggests that a primary function of these transient actin puncta is to assist in the late stages of clathrin-coated pit internalization.

3.2.8 Epsin null cells display reduced association of actin puncta with clathrin puncta on the plasma membrane.

To determine whether epsin is important for this interaction between actin and clathrin, we co-expressed clathrinRFP and LimEΔcoilGFP in epsin null cells. Using

TIRF microscopy, we found that, similarly to wild-type cells, many clathrin puncta in epsin null cells also associated with actin prior to internalization (Figure 3.4E). Nevertheless, the clathrin puncta persisted at the membrane in the epsin null cells much longer than in wildtype cells before internalization (Figure 3.4, B and G). On the other hand, slightly fewer actin puncta in epsin null cells associated with clathrin. 67% of actin puncta in epsin null cells associated with clathrin puncta, compared with 72% of actin puncta in wild-type cells (Figure 3.4F). Surprisingly, despite the increased mobility of actin puncta in epsin null cells, 95% of the laterally mobile actin puncta in epsin null cells associated with clathrin puncta. This suggested that the abnormal mobility of the actin puncta was not a factor in the decreased association of actin with clathrin puncta in epsin null cells.

3.2.9 Loss of Hip1r affects clathrin and actin dynamics

The increased persistence at the membrane of clathrin puncta, the increased mobility and abnormal morphology of actin puncta, and the decreased association of actin puncta with clathrin puncta indicate that epsin regulates the productive interaction of actin with clathrin-coated pits. We hypothesized that the mechanism of this regulation involved the recruitment of Hip1r to the membrane. If this is the case, Hip1r null cells should display the same defects in clathrin and actin dynamics.

To determine if Hip1r affected clathrin dynamics, we examined Hip1r null cells expressing clathrinRFP under TIRF microscopy. In Hip1r null cells, clathrin formed discrete puncta on the plasma membrane similar to wild-type cells. However, 57% of clathrin puncta in Hip1r null cells identified at the beginning of timelapse capture persisted at the membrane for more than 60 seconds, compared to only 3% of clathrin puncta in wild-type cells (Figure 3.4, A and B). Furthermore, 40% of clathrin

puncta in Hip1r null cells persisted longer than 120 seconds. This is similar to the behavior of clathrin puncta in epsin null cells, but more severe, indicating that Hip1r is fundamentally involved in clathrin dynamics at the plasma membrane.

To determine if Hip1r also influences actin dynamics at the cell surface, we expressed LimEΔcoilGFP in Hip1r null cells to label dynamic actin in the cell. Under TIRF microscopy, actin formed transient puncta in Hip1r null cells similar to wild-type cells (Figure 3.4C). However, 66% of actin puncta in Hip1r null cells were elongated and laterally mobile, compared to 43% in epsin null cells and only 14% in wild-type cells (Figure 3.4D). Thus Hip1r has a more pronounced effect on actin dynamics at the membrane than epsin.

To determine if the absence of Hip1r affected the association of clathrin and actin in living cells, we co-expressed both clathrinRFP and LimEΔcoilGFP in Hip1r null cells and examined them with TIRF microscopy. As with wild-type cells, actin frequently associated with clathrin puncta just before the loss of clathrin signal at the membrane (Figure 3.4E). However, a lower percentage, 59%, of actin puncta in Hip1r null cells associated with clathrin, compared to 72% in wild-type cells (Figure 3.4F). Despite the decrease in actin puncta associated with clathrin in the mutant cells, all clathrin puncta that did associate with actin internalized immediately. These results indicate that, like epsin, Hip1r is also involved in the coupling of actin to clathrin. The actin and clathrin phenotypes exhibited by Hip1r null mutants were more severe that those of epsin mutants, supporting the idea that epsin may regulate actin and clathrin through Hip1r.

3.2.10 PI(4,5)P_2-binding ability of the epsin ENTH domain is required for Hip1r localization and phosphorylation

Epsin is required for the phosphorylation of Hip1r and its localization to the plasma membrane. We have previously shown that the ENTH domain of epsin is both necessary and sufficient to rescue these deficiencies (Repass et al., 2007). The function of the ENTH domain can be abolished by point mutations in residues R65 and K78. These mutations disrupt the ability of the ENTH domain to bind to PI(4,5)P_2. An epsin$^{R65A/K78}$ mutant cannot localize within clathrin pits on the plasma membrane and is unable to rescue epsin-related phenotypic deficiencies (see Chapter 2).

To determine whether the R65A/K78A mutations affect the ability of the ENTH domain to rescue Hip1r localization, we expressed epsin^{WT}GFP or epsin$^{R65A/K78A}$GFP in an epsin null background and immunostained for Hip1r (Figure 3.5A). As we observed previously, Hip1r forms discrete puncta on the plasma membrane of wild-type cells, but does not associate with the plasma membrane of epsin null cells. Expressing epsin^{WT}GFP in epsin null cells led to the formation of Hip1r puncta at the plasma membrane, a pattern indistinguishable from wild-type cells (Figure 3.5A, top row). On the other hand, expression of epsin$^{R65A/K78A}$GFP, which does not bind to PI(4,5)P_2 and does not localize to clathrin pits, did not restore Hip1r localization to the plasma membrane. Instead, Hip1r formed cytoplasmic puncta similar to the distribution in epsin null cells (Figure 3.5A, second row, compare Figure 3.3A), indicating that epsin$^{R65A/K78A}$GFP is not capable of restoring the membrane localization of Hip1r in epsin null cells.

Epsin is also required for the phosphorylation of Hip1r. To determine whether the epsin$^{R65A/K78}$ mutant can restore Hip1r phosphorylation, we prepared lysates of

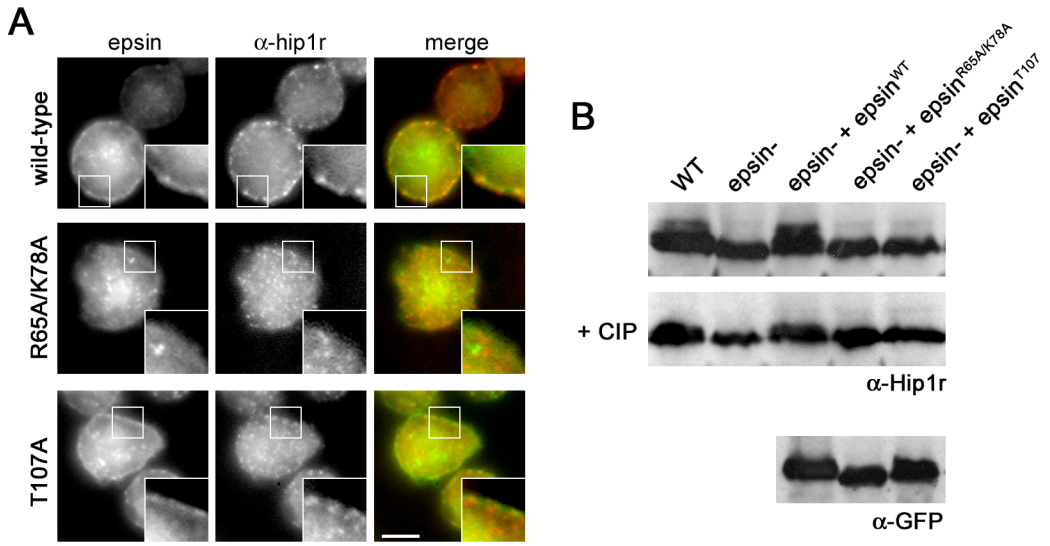

Figure 3.5. Epsin$^{R65A/K78A}$ and epsin^{T107A} cannot rescue the localization or phosphorylation of Hip1r in epsin null mutants.

(A) Epsin$^{R65A/K78A}$ and epsin^{T107A} cannot rescue the localization of Hip1r. Epsin null cells expressing epsin^{WT}GFP (**wild-type**, top row), epsin$^{R65A/K78A}$GFP (**R65A/K78A**, center row), or epsin^{T107A}GFP (**T107A**, bottom row) were fixed an immunostained with anti-Hip1r antibodies. Bar, 5μm. (B) Epsin$^{R65A/K78A}$ and epsin^{T107A} cannot restore the phosphorylated species of Hip1r. (top row) Wildtype cells (**WT**), epsin null cells (**epsin-**), and epsin null cells expressing epsin^{WT}GFP (**epsin- +epsin^{WT}**), epsin$^{R65A/K78A}$GFP (**epsin- +epsin$^{R65A/K78A}$**), or epsin^{T107A}GFP (**epsin- +epsin^{T107A}**) were analyzed by Western blot probed with anti-Hip1r antibodies. (center row) Lysates were treated with calf intestinal alkaline phosphatase (**+CIP**) before analysis. (bottom row) Lysates of epsin null cells expressing epsin^{WT}GFP, epsin$^{R65A/K78A}$GFP, or epsin^{T107A}GFP were analyzed by Western blot probed with anti-epsin antibodies to confirm expression of epsin mutant constructs.

epsin null cells expressing wild-type epsinGFP and epsin$^{R65A/K78}$GFP in epsin null cells and analyzed them by immunoblot using anti-Hip1r antibodies (Figure 3.5B). Blots of lysates from wild-type cells contained two species of Hip1r that appeared as a doublet on the blot (Figure 3.5B, first lane). The upper band of this doublet was absent in lysates treated with phosphatase, indicating that the upper band of Hip1r represented a phosphorylated species of Hip1r (Figure 3.5B, bottom row). This phosphorylated band was absent in blots of epsin-null lysates. Expressing epsin^{WT}GFP in epsin-null cells restored the phosphorylated species of Hip1r. However, immunoblots of lysates from epsin null cells expressing epsin$^{R65A/K78}$GFP did not display the upper band of the Hip1r doublet, indicating that epsin$^{R65A/K78}$ cannot restore the phosphorylated form of Hip1r in epsin null mutants (Figure 3.5B). Thus the ability of the ENTH domain to bind PI(4,5)P_2 is important for epsin to facilitate the recruitment of Hip1r to the membrane and induce the phosphorylation of Hip1r.

3.2.11 The epsin ENTH domain regulates Hip1r via a residue independent of PIP(4,5)P_2 binding

A second mutation in the ENTH domain, T107A, retains the ability to bind to PI(4,5)P_2 and to localize to clathrin-coated pits on the plasma membrane, but fails to restore the ability of epsin to rescue the phenotypic deficiencies of epsin null cells (see Chapter 2). To determine if the epsin^{T107A} mutant could rescue Hip1r localization in epsin null cells, we expressed epsin^{T107}GFP in epsin null cells and immunostained with anti-Hip1r antibodies (Figure 3.5A). Although epsin^{T107}GFP formed puncta on the membrane, Hip1r did not form membrane puncta in epsin null cells expressing epsin^{T107}GFP. Instead, Hip1r localized to the cytoplasm, as in epsin null cells (Figure

3.5A, bottom row), demonstrating that epsin^{T107}GFP was not capable of restoring the membrane localization of Hip1r in epsin null cells.

To determine whether epsin^{T107} was able to restore the phosphorylation of Hip1 in epsin null mutants, we analyzed lysates of epsin null cells expressing epsin^{T107}GFP by immunoblot. Probing with anti-Hip1r antibodies revealed that epsin-null cells expressing epsin^{T107}GFP contain only the unphosphorylated species of Hip1r (Figure 3.5B), indicating that the T107A mutation in epsin abolished the ability of epsin to facilitate the phosphorylation of Hip1r. Thus, residue T107 represents an essential function of the ENTH domain, separate from binding PI(4,5)P_2, that is required for the recruitment and phosphorylation of Hip1r.

3.2.12 Epsin^{T107A} localized to clathrin pits in similar proportions to epsin^{WT}

The inability of epsin^{T107} to rescue epsin null phenotypes, including the phosphorylation and membrane recruitment of Hip1r, could be explained by a more subtle defect in the association of epsin^{T107} with clathrin coated pits. To test this hypothesis, we co-expressed either epsin^{WT}GFP or epsin^{T107}GFP with clathrinRFP in an epsin null background and immunostained for the clathrin adaptor AP2 (Figure 3.6A). When examined under epifluorescence microscopy, 57% ± 4% (mean ± SE, n = 16 cells) of epsin^{WT}GFP puncta on the plasma membrane colocalized with both clathrin and AP2. A similar percentage of epsin^{T107}GFP, 55% ± 4%, also colocalized with both clathrin and AP2 (Figure 3.6,A and C), suggesting that the T107A mutation does not affect the ability of epsin to incorporate into clathrin-coated pits on the plasma membrane.

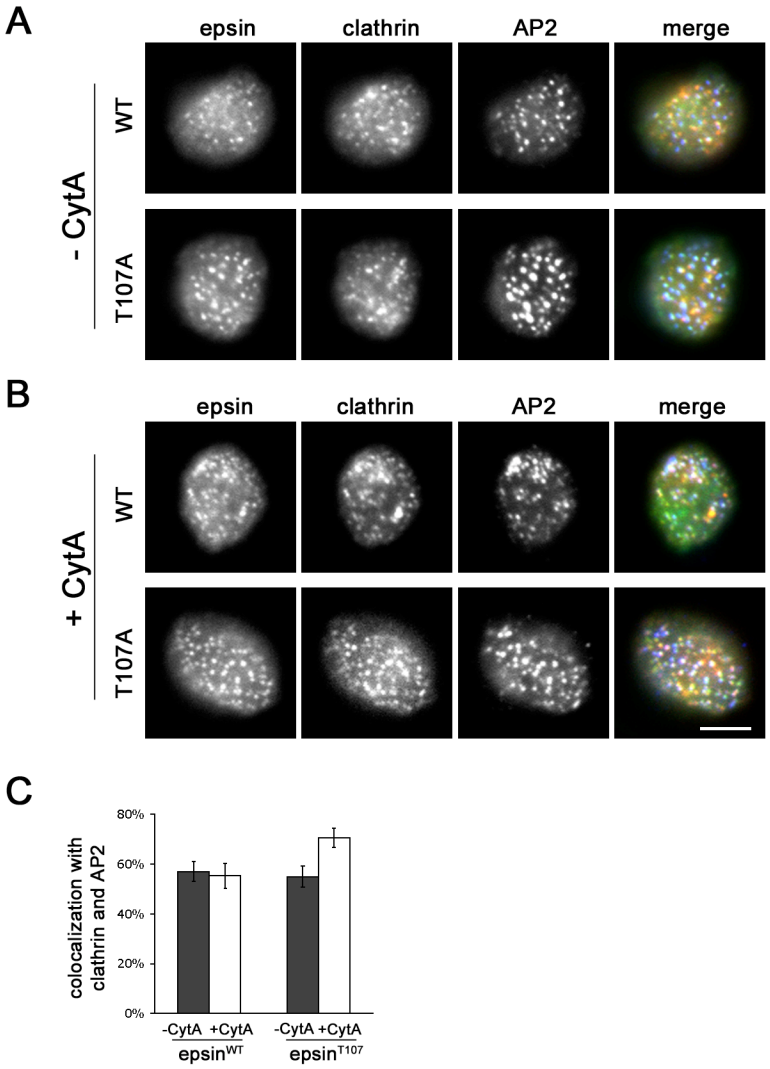

Figure 3.6. Epsin^{T107A} localizes to clathrin pits that contain AP2 and is sensitive to cytochalasin A treatment.

(A) Epsin null cells expressing either epsin^{WT}GFP (**WT**) or epsin^{T107A}GFP (**T107A**) and co-expressing clathrinRFP were fixed and immunostained with anti-AP2 antibodies and the surface of the cell imaged by epifluorescence microscopy. (B) Epsin null cells expressing either epsin^{WT}GFP (**WT**) or epsin^{T107A}GFP (**T107A**) and co-expressing clathrinRFP were treated with cytochalasin A (**+ CytA**), then fixed and immunostained with anti-AP2 antibodies. Surface of the cell imaged by epifluorescence microscopy. Bar, 5μm. (C) Quantification of colocalization between clathrin, AP2, and either Epsin^{WT} or Epsin^{T107A} with and without cytochalasin A treatment. Error bars are standard error.

3.2.13 Epsin^{T107A} localization is sensitive to cytochalasin A treatment

Because epsin facilitates the recruitment of Hip1r to the plasma membrane, and this recruitment is sensitive to perturbations in actin dynamics by cytochalasin A treatment, we asked whether the T107A mutation affected the localization of epsin when treated with cytochalasin A. Epsin null cells co-expressing either epsin^{WT}GFP or epsin^{T107}GFP with clathrinRFP were treated with cytochalasin A and immunostained for AP2 (Figure 3.6B). Upon examination by epifluorescence microscopy, we found the proportion of epsin^{WT}GFP puncta that simultaneously associated with clathrin and AP2 after cytochalasin A treatment was 55% ± 4% (n = 5 cells), close to the percentage of colocalization in untreated cells. On the other hand, the percentage of epsin^{T107A}GFP that colocalized with both clathrin and AP2 increased with cytochalasin A treatment, from 55% to 71% ± 4% (Figure 3.6C). This suggests that the T107A mutation increases the sensitivity of epsin to changes in actin dynamics. However, more cells will need to be analyzed to confirm the statistical significance of this difference.

3.2.14 Epsin^{T107A} failed to rescue the dynamic actin defects of epsin null cells

The T107A mutation did not interfere with the ability of epsin to associate with clathrin pits on the membrane, but it did interfere with the ability of epsin to facilitate Hip1r recruitment and phosphorylation. To determine if the T107A mutation also influences the ability of epsin to regulate actin puncta at the cell surface, we co-expressed epsin^{T107}GFP and LimEΔcoilRFP in epsin null cells and examined the LimEΔcoil-labels actin puncta under TIRF microscopy. We found that 43% of actin

puncta at the cell surface were misshapen and laterally mobile, similar to epsin null mutants. Thus the T107A mutation, which abolishes the ability of epsin to facilitate the recruitment and phosphorylation of Hip1r, does not rescue the defects in actin dynamics of epsin null cells.

3.3 DISCUSSION

Interactions between clathrin adaptors are an important part of the organization and regulation of a clathrin-coated pit. We have established that both Hip1r and epsin are important for the productive coupling of actin to a clathrin pit. We have shown that epsin is important for Hip1r function and identified residues in the ENTH domain critical for this regulation. We propose that epsin regulates the actin cytoskeleton during clathrin-mediated endocytosis by facilitating the recruitment and phosphorylation of Hip1r.

3.3.1 Actin and clathrin dynamics in *Dictyostelium*

Actin plays an important and conserved role in clathrin-mediated endocytosis. In mammals and yeast, treatment with actin depolymerizing drugs arrests clathrin pits on the plasma membrane. We found a similar effect in *Dictyostelium*. The accumulation of clathrin puncta on the membrane in *Dictyostelium* cells treated with cytochalasin A suggests that inhibiting actin polymerization impairs the maturation and scission of a coated pit.

Examining the actin and clathrin dynamics at the membrane confirmed that actin functions in the late stages of clathrin-mediated endocytosis. We found that actin puncta associated with clathrin just before the internalization of the coated pit.

These localized bursts of polymerization may provide the mechanical force necessary to complete scission. However, a large population of clathrin puncta internalized without an associated actin puncta. This may indicate that there are redundant mechanisms for coated-pit internalization. Alternatively, the clathrin puncta disappearing without actin polymerization may be disassembling instead of internalizing. It will be important to analyze the puncta with simultaneous TIRF and epifluorescence microscopy to more accurately identify puncta internalizing from the membrane. On the other hand, a large majority of actin puncta associated with clathrin, indicating that a primary function of these puncta is to participate in coated pit internalization. Surprisingly, electron micrographs of LimEΔcoil-labeled actin structures at the membrane do not show evidence of clathrin lattices (Bretschneider et al., 2004). This may be due to the transient nature of the actin puncta and the rapid dissociation of associated clathrin vesicles from the membrane, making it difficult to find clathrin and actin associated structures in fixed cells.

3.3.2 Epsin regulates Hip1r

Epsin is specifically required for the localization and phosphorylation of Hip1r. This dependence is unique among clathrin adaptors, as AP2, AP180, auxilin, and eps15 mutants all contain phosphorylated Hip1r (Graham and O'Halloran, unpublished results). These localization and phosphorylation requirements are somewhat separable. While Epsin is required for the phosphorylation of Hip1r under all circumstances tested, epsin appears to only be required for the recruitment and/or stabilization of Hip1r to the plasma membrane in the presence of dynamic actin. Furthermore, Hip1r associates with clathrin-coated pits in the absence of epsin, but only when actin polymerization is inhibited. This may indicate that, while epsin

promotes the association of Hip1r with the membrane, actin counters this activity and destabilizes the association of Hip1r with the membrane.

Epsin may accomplish this stabilization through the phosphorylation of Hip1r. Although Hip1r can be recruited to the membrane in epsin null cells if actin is depolymerized, Hip1r remains unphosphorylated. This suggests that phosphorylation of Hip1r occurs through an epsin-dependent pathway. One explanation is that Hip1r localizes to the membrane via the $PI(4,5)P_2$-binding ANTH domain and associates with clathrin via its central coiled-coil domain. This interaction with clathrin-coated pits may be transient and unstable without subsequent phosphorylation. Epsin, which targets to clathrin-coated pits independently of Hip1r, may be necessary to recruit an intermediate kinase to the coated pit, which subsequently phosphorylates and stabilizes Hip1r.

3.3.3 ENTH domain residues critical for epsin to regulate Hip1r

We have previously shown that the ENTH domain of epsin is sufficient to restore the membrane localization and phosphorylation of Hip1r in epsin null cells (Repass et al., 2007). Our mutant analysis of the ENTH domain revealed that the ability to bind $PI(4,5)P_2$ is central to the function of the ENTH domain. However, $PI(4,5)P_2$-binding by the ENTH domain is not sufficient to rescue the Hip1r defects in epsin null cells.

The mutation in residue T107 of the ENTH affected the function of epsin without affecting epsin localization to clathrin-coated pits. The isolated ENTH domain is sufficient to restore Hip1r localization and phosphorylation without localizing to clathrin puncta (Repass et al., 2007). Thus localization to clathrin-coated pits is not sufficient, or even required, for epsin to interact with Hip1r. This is

surprising considering the high degree of colocalization between epsin, Hip1r and clathrin on the membrane of wild-type cells.

In yeast, the analogous residue to T107 is critical for the ENTH domain to bind a GAP for cdc42, which then remodels the actin cytoskeleton at the cortex. The *Dictyostelium* genome does not contain a clear cdc42 homolog. *Dictyostelium* does contain many Rho and Rac GTPases with their associated GAPs. It will be important to elucidate the mechanism by which this residue contributes to epsin function.

3.3.4 Epsin regulates actin through Hip1r

Although the ENTH domain of epsin has been implicated in general actin dynamics in yeast (Aguilar et al., 2006; Wendland et al., 1999), epsin has not been shown previously to affect actin dynamics during clathrin-mediated endocytosis. We have shown that, in *Dictyostelium*, epsin is important for the productive coupling of actin to the clathrin machinery. Without epsin, actin puncta become disorganized and less able to associate with clathrin. The Hip1r mutant has similar phenotypes, suggesting the two adaptors function in the same pathway. Epsin null mutants fail to localize Hip1r properly, and the ENTH T107 mutant fails to rescue Hip1r and fails to rescue the disorganized actin puncta. Therefore, we propose that epsin modulates actin dynamics at clathrin-coated pits by facilitating the recruitment and phosphorylation of Hip1r.

3.3.5 How does Hip1r regulate clathrin and actin dynamics?

Hip1r contains modules for binding clathrin, actin, and the plasma membrane, leading many to suggest that Hip1r forms a molecular bridge between clathrin and

actin during clathrin-mediated endocytosis (Engqvist-Goldstein et al., 1999; Engqvist-Goldstein et al., 2001; Newpher and Lemmon, 2006). This idea has been substantiated by *in vitro* binding assays demonstrating that Hip1r can bind to actin and clathrin simultaneously (Engqvist-Goldstein et al., 2001). In *Dictyostelium*, actin puncta in Hip1r null cells did not associate with clathrin as frequently as in wild-type cells. Our results indicate that Hip1r facilitates the coupling of actin polymerization with clathrin-coated pits, consistent with a role in bridging actin and clathrin structures. Our results also found that internalization of clathrin was impaired in Hip1r null cells. Clathrin puncta remained static on the membrane for prolonged periods of time. This is very similar to the phenotype seen in sla2p mutants, the yeast homolog of Hip1r, which also show stabilized clathrin puncta on their membranes (Newpher et al., 2005).

Reducing Hip1r expression in mammalian cell culture via RNAi has been reported to cause non-productive couplings between clathrin and actin. In that study, actin associated with clathrin, but instead of internalizing, the clathrin pit remained on the cell surface while the actin "tail" waved in the cytoplasm (Engqvist-Goldstein et al., 2004). This led to the conclusion that Hip1r negatively regulated the polymerization of actin at endocytic sites. We found a different behavior for actin puncta in our *Dictyostelium* Hip1r null cells. All clathrin puncta associating with actin puncta in both wild-type and Hip1r null cells subsequently internalized. However, we did observe abnormal movement and polymerization of the actin puncta, and many actin puncta which did not associate with clathrin. Thus Hip1r may inhibit the inappropriate polymerization of actin at sites other than clathrin pits.

Chapter 4: Conclusions and Future Directions

In this work, we identified and characterized the *Dictyostelium* homolog of the clathrin-associated protein, epsin,. We found that the determinants for epsin localization are distinct and separable from the determinants that contribute to epsin function. We demonstrated that the functionality of the ENTH domain extends beyond binding $PI(4,5)P_2$ and includes facilitating the phosphorylation and membrane recruitment of Hip1r and regulating spore morphology. In addition, we described the dynamics between clathrin and actin in late stages of clathrin pit internalization. We found that both Hip1r and epsin promote the productive coupling of actin to the clathrin pit for efficient internalization. Our results broaden the role for epsin and the ENTH domain during clathrin-mediated endocytosis and highlight the dynamic, multifunctional characteristics of clathrin adaptors at the plasma membrane.

4.1 $PI(4,5)P_2$ BINDING IS FUNDAMENTAL TO ENTH DOMAIN FUNCTION

The ability to bind $PI(4,5)P_2$ is the most well defined characteristic of the ENTH domain. This ability is thought to facilitate unique membrane-bending properties of the ENTH domain. When the ENTH domain binds to $PI(4,5)P_2$, an $\alpha 0$-helix is induced that inserts into the membrane, inducing curvature (Ford et al., 2002; Kweon et al., 2006; Stahelin et al., 2003). We found that the membrane-binding capacity of the ENTH domain is critical for the function of epsin in *Dictyostelium*. Without the ENTH domain, the carboxy-terminal portion of epsin cannot localize to clathrin-coated pit on the membrane or cannot fully rescue epsin null phenotypic

deficiencies. Similar results are seen when the ENTH domain is mutated so that it no longer binds $PI(4,5)P_2$.

While the ENTH domain alone is not sufficient to localize epsin to clathrin structures on the membrane, it retains significant function. The ENTH domain alone can rescue both the spore morphology defect and the cytokinesis defect of epsin-null cells. The isolated ENTH domain is also sufficient to target Hip1r to the plasma membrane. All these functions require the capacity of the ENTH domain to bind to $PI(4,5)P_2$.

This fundamental requirement for binding $PI(4,5)P_2$ suggests that any other functions of the ENTH domain have evolved to take advantage of the membrane localization and/or binding of the primordial ENTH domain. The ability of the ENTH domain to induce membrane curvature may be similarly important to other functions of the ENTH domain. If the ability to induce curvature were disrupted without affecting affinity for $PI(4,5)P_2$, would the other functions of the ENTH domain be disrupted? This could be accomplished using point mutations that would ablate the capacity to curve the membrane, but leave $PI(4,5)P_2$ binding intact (Ford et al., 2002).

Although binding to $PI(4,5)P_2$ appears to be fundamental to ENTH domain function, there may be other functions of the ENTH domain that do not require $PI(4,5)P_2$ binding. The ENTH domain has been implicated in gene transcription (Hyman *et al.*, 2000; Vecchi *et al.*, 2001). Although the ENTH domain cannot initiate transcription off a model promoter as efficiently as the ANTH domain, it nonetheless displays a nuclear localization (Vecchi *et al.*, 2001). The ENTH domain may have an as-yet-undefined role in gene regulation independent of binding $PI(4,5)P_2$.

4.2 ENTH MODERATES INTERACTION BETWEEN EPSIN AND CLATHRIN

Our results using the PH:epsin$_{253\text{-}677}$ chimera demonstrated that, once targeted to the membrane, the carboxy-terminal section of epsin could bind clathrin to the extent that it disrupted clathrin function. The potent ability of the carboxy-terminal segment of epsin to bind and recruit clathrin is only evident when tethered to the plasma membrane, as the carboxy-terminal fragment alone did not cause aberrant localization of clathrin. Restricting the clathrin-binding function of epsin to the plasma membrane may prevent unregulated clathrin assembly at inappropriate locations in the cell. Furthermore, the disruption of clathrin distribution and function associated with expression of the PH:epsin$_{253\text{-}677}$ chimera also demonstrated a new function for the ENTH domain: the ability to modulate the clathrin-binding capacity of epsin.

The *Dictyostelium* ENTH domain contains two different clathrin-binding motifs, and epsins from metazoans also contain AP2-binding motifs within their ENTH domains. Potentially, as membrane curvature increases, changes to the conformation of the ENTH domain may alter the accessibility of these clathrin-binding sites, affecting the affinity of epsin for clathrin. The clathrin-binding motifs in the ENTH domain and the carboxy-terminal region of epsin may compete and destabilize the interaction between epsin and clathrin, allowing epsin to release clathrin as the pit matures. In addition, the affinity of the ENTH domain itself for PI(4,5)P_2, which was measured *in vitro* (Itoh *et al.*, 2001), could change *in vivo* depending on the degree of curvature of the membrane, thereby modulating the association of epsin with the invaginating coated pit.

4.3 EXPLORING THE SIGNIFICANCE OF THE ENTH T107 RESIDUE

We found the ENTH domain was critical to epsin localization and function. We also found that the function of the ENTH domain can be separated from its localization. The point mutant epsin^{T107A} was able to localize to clathrin-coated pits the plasma membrane and did not interfere with clathrin function. However, this point mutation did compromise the ability of epsin^{T107A} to rescue phenotypic deficiencies of epsin null cells, including spore morphology, Hip1r phosphorylation and localization, and actin dynamics at the plasma membrane.

Although we were able to determine that the epsin^{T107A} mutant did not rescue the disorganized actin polymerization in epsin null cells, we could not assess whether epsin^{T107A} influenced the association of actin with clathrin. This was due to the limitations of only having two colors of fluorescent tags available to us. This may be overcome by adapting a third color of fluorescent protein, such as Azurite/EBFP2 (Kremers et al., 2007), for expression in *Dictyostelium*. This would allow for expression of three fluorescently labeled constructs simultaneously, so that we could image clathrin, actin, and epsin together in living cells.

In yeast, the analogous residue to T107 is part of a patch on the ENTH domain that bind to the cdc42 GAP proteins Rga1 and Rga2 (Aguilar et al., 2006). Mutations in this patch of residues impair the function of the yeast ENTH domain (Aguilar et al., 2006). The T107 residue in the *Dictyostelium* ENTH domain may be involved in a similar pathway. However, the *Dictyostelium* genome does not contain a close homolog to Rga1 or Cdc42. Instead, *Dictyostelium* has a large array of Racs and Rhos as small GTPase regulators of actin. The *Dictyostelium* ENTH domain may bind GAP proteins for one of these GTPases. Identifying the mechanism by which residue T107

affects ENTH function will be key in understanding the broader mechanism of interaction between epsin and Hip1r.

4.4 ENTH REQUIRED FOR HIP1R LOCALIZATION AND FUNCTION

The ENTH domain is required for the phosphorylation and membrane recruitment of Hip1r (Repass et al., 2007). This requirement is specific for epsin, as Hip1r is phosphorylated and recruited to the membrane in AP2, clathrin light chain, clathrin heavy chain, and AP180 null mutants (Repass, Graham, and O'Halloran, unpublished data). A clathrin-coated pit is formed from a network of adaptors and interactions, so it is surprising that Hip1r function would be so specifically dependent on the presence of one other adaptor.

There is much about the mechanism of this relationship that is not understood. Epsin and Hip1r have no motifs or domains that are predicted to interact with each other based on sequence analysis. One mechanism of interaction might be through a common interaction with clathrin. Epsin binds to clathrin heavy chain (see chapter 2), while Hip1r has been shown in other systems to interact with clathrin light chain. Thus the assembled clathrin triskelion might form a molecular bridge between these two adaptors. However, Hip1r is still phosphorylated and recruited to the membrane in both clathrin light chain and clathrin heavy chain mutants, arguing against this possibility.

One clue to the mechanism of this relationship is that Hip1r localization and phosphorylation are correlated with each other. Hip1r is neither recruited to the membrane nor phosphorylated in epsin null cells. Mutant epsin constructs that fail to rescue phosphorylation also fail to rescue Hip1r localization. The only experimental condition where Hip1r phosphorylation was separated from membrane recruitment

was when epsin null cells were treated with cytochalasin A. Under these conditions of impaired actin polymerization, Hip1r was able to form membrane puncta that associated with clathrin without becoming phosphorylated. This indicates that membrane localization is not sufficient for phosphorylation. Rather, epsin may be facilitating the phosphorylation of Hip1r, which then stabilizes Hip1r at the membrane.

An important step towards elucidating the mechanism of the Hip1r/epsin relationship will be to identify the phosphorylation site on Hip1r. This would allow the generation of Hip1r point mutants that either prevent or mimic phosphorylation, which could then be used to study the effect of phosphorylation on Hip1r function. Identification of the phosphorylation site could also reveal a consensus site for a specific kinase, suggesting signaling pathways and regulation to test, These steps are important to determine the intermediary steps that allow epsin to facilitate the phosphorylation of Hip1r..

4.5 EPSIN AND HIP1R IN SPORE DEVELOPMENT

We have described a role for epsin in *Dictyostelium* spore development. Epsin null cells develop spores with an abnormal, round morphology. It is unclear whether spore morphology is a clathrin related process, as clathrin heavy chain mutants arrest in development before the formation of spores. Nonetheless it is clear that this developmental process involves only a specialized subset of clathrin adaptors. While epsin and Hip1r are important for the formation of oblong spores (Repass et al., 2007), other adaptors such as AP2 and AP180 are not (Wen and O'Halloran, unpublished results). Interestingly, the epsin point mutant T107A that did not rescue Hip1r localization or phosphorylation also did not rescue spore morphology defect in

epsin null cells, despite localization to clathrin-coated pits. This suggests that there may be a link between the membrane recruitment and phosphorylation of Hip1r and the formation of oblong spores.

How might these adaptors function during spore development? One possibility is that epsin and Hip1r function in membrane trafficking events in prespore cells. Early in the *Dictyostelium* developmental cycle, cells fated to become spores form prespore vesicles (Hohl and Hamamoto, 1969). This vesicle contains many of the proteins necessary for the formation of the spore coat (Srinivasan et al., 2000). The origins of this organelle are unknown. Epsin and Hip1r may function in trafficking events that modify an existing organelle, such as the contractile vacuole, into the prespore vesicle. Proteomic analysis of fully formed prespore vesicles did not identify clathrin or any clathrin adaptors (Srinivasan et al., 2001). Thus, if epsin and Hip1r are involved in the genesis of this organelle, they are not present in significant quantities after the prespore vesicle has formed.

Upon receipt of a specific and unknown signal, all the prespore cells secrete the contents of their prespore vesicles and fully differentiate into spores. Very little is known about the mechanisms that control this event. The coordinated nature of the secretion suggests that the prespore cells receive an extracellular signal. Epsin and Hp1r may be involved in the appropriate processing of the activated membrane receptor for this signal.

Separate from the formation of the prespore vesicle, epsin and Hip1r may be more directly involved in the generation of oblong spores. Starting in mid-culmination, developing *Dictyostelium* spores begin to form large, thick actin tubules composed of three or more actin filaments. These actin rods persist through spore maturation (Sameshima et al., 2001). Cells lacking these rods form round spores

(Sameshima et al., 2000). Epsin and Hip1r may be directly involved in the organization of these actin rods, or in processing the signals that are required to induce the formation of these rods. One candidate membrane protein is SrfA, a transmembrane protein required for the formation of these rods (Escalante et al., 2004). SrfA null mutants also form round spores (Escalante et al., 2004). Epsin and Hip1r null mutants may have malformed actin rods and/or mislocalized SrfA, which may account for the spore morphology defects in these cells.

4.6 EPSIN ENTH DOMAIN AFFECTS CLATHRIN AND ACTIN DYNAMICS THROUGH HIP1R

Dynamic actin plays an important role during clathrin-mediated endocytosis in *Dictyostelium*. We found that, similar to mammalian cells, a small burst of actin polymerization accompanies the internalization of a clathrin puncta (Merrifield et al., 2002). When we impaired the polymerization of actin, there was an accumulation of clathrin pits on the plasma membrane. In mutants with disorganized actin polymerization, we found an increase in the persistence of clathrin puncta on the membrane. This indicates that actin is involved in the later stages of clathrin-mediated endocytosis, such as invagination and scission. In yeast, actin primarily affects clathrin pit mobility and turnover, with little effect on clathrin assembly (Newpher et al., 2005). In mammalian cells, actin has been implicated in all stages of clathrin pit maturation (Merrifield et al., 2005; Yarar et al., 2005).

Our results suggest that the actin and clathrin dynamics of *Dictyostelium* are closer to yeast, with actin involved predominantly in later stages. However, if this is the case, why are there not more clathrin dots on the membrane of mutants with disorganized actin? One possibility is that the rates of clathrin pit assembly are also

slower in these mutants. Another possibility is that these null mutants have adapted to initiate fewer clathrin pits at membrane so as not to sequester all clathrin at the cell surface.

This work is the first to describe defects in the coupling actin polymerization to clathrin-coated pits in the absence of epsin. Suggestions of the involvement of epsin in actin organization have come from studies in yeast, where epsin null mutants have enlarged and disorganized actin patches that do not polarize properly during cell division (Wendland et al., 1999). Yeast epsins are phosphorylated by actin-regulated kinases (Watson et al., 2001) and may modify the actin cytoskeleton by regulating the activity of cdc42 (Aguilar et al., 2006). However, the dynamics of clathrin and actin at the plasma membrane have not been studied in yeast epsin null mutants.

We found that in *Dictyostelium* epsin mutants, the small bursts of actin polymerization are elongated and have increased lateral mobility. Clathrin pits persist longer at the membrane before associating with an actin puncta and internalizing. These defects are reminiscent of actin and clathrin defects in mammalian and yeast Hip1r mutants. Because epsin is required for the phosphorylation and membrane recruitment of Hip1r, we hypothesized that the actin defects we found in epsin mutants were caused by the mislocalization and/or nonphosphorylation of Hip1r. We found that Hip1r mutants displayed the similar clathrin and actin defects, only more severe. Furthermore, mutations in the ENTH domain that fail to rescue deficiencies associated with Hip1r also fail to rescue the actin defects of epsin null cells.

POTENTIAL MECHANISMS FOR HIP1R REGULATION OF ACTIN

There are many possibilities for how Hip1r may be involved in clathrin and actin dynamics at the plasma membrane. Hip1r contains domains that bind the

membrane, clathrin, and actin (Chen and Brodsky, 2005; Engqvist-Goldstein et al., 1999; Sun et al., 2005; Ybe et al., 2007a). Hip1r also forms a homodimer (Engqvist-Goldstein et al., 2001), allowing it to bind more than one molecule of clathrin and/or actin simultaneously. From this structural analysis, the simplest explanation for the function of Hip1r is that Hip1r forms a molecular bridge between clathrin and actin. This would explain our finding that clathrin puncta persist longer at the plasma membrane before associating with actin in Hip1r null mutants.

Mammalian and yeast Hip1r has been shown to negatively regulate the polymerization of actin (Le Clainche *et al.*, 2007). In these systems, the loss of Hip1r leads to treadmilling actin "tails" at the membrane. In *Dictyostelium*, Hip1r null cells display elongated and laterally mobile actin puncta. Thus, one function of Hip1r may be to facilitate localized, efficient polymerization at the site of endocytosis. However, there is an important difference between mammalian and *Dictyostelium* Hip1r function. In mammalian cells lacking Hip1r, actin forms a more stable association with clathrin that does not lead to internalization. By contrast, *Dictyostelium* cells lacking Hip1r continue to form productive associations between clathrin and actin. While there is a delay before actin puncta form in association with clathrin, it nonetheless results in the internalization of the clathrin pit.

Hip1r has also been shown to negatively regulate cortactin, preventing the polymerization of actin until just before scission (Le Clainche *et al.*, 2007). Although the *Dictyostelium* genome does not contain a clear cortactin homolog, Hip1r may regulate other important actin-associated proteins to temporally control the polymerization of actin at sites of endocytosis.

Visualizing the dynamics of Hip1r with respect to actin and clathrin will be an important next step in understanding how Hip1r functions in this process.

Furthermore, once the phosphorylation site of Hip1r as been identified, the constructs that mimic the phosphorylated or dephosphorylated form of Hip1r can be generated to determine how the phosphorylation state of Hip1r affects its ability to regulate actin and clathrin dynamics, and how epsin may be involved in this process.

APPENDICES

Appendix A: Materials and Methods

Strains and cell culture

Dictyostelium discoideum strains included Ax2, an axenic wild-type strain, 10G10 and 5B4, epsin null strains derived from Ax2 (described below), 6A5, an α-adaptin null line derived from Ax2 (kind gift of Y. Wen), 5E2, a CHC null line derived from Ax2 (Niswonger and O'Halloran, 1997b), and 2A1, a CLC null strain derived from NC4A2 (Wang *et al*., 2003). Cells were cultured on tissue culture plates with HL-5 medium (Sussman, 1987) supplemented with 60U/mL penicillin and 60μg/mL streptomycin (Invitrogen, Inc., Carlsbad, CA) at 18°C. Null cells grown under selection were supplemented with 5μg/mL blasticidin (ICN Biomedicals, Irvine, CA), and cells carrying expression plasmids were supplemented with 20μg/mL G418 (geneticin, Gibco-BRL, Invitrogen, Inc., Carlsbad, CA).

Targeted replacement of epnA gene in *Dictyostelium discoideum*

Genomic sequence upstream of *epnA* was PCR-amplified using 5' TTAAAAAAGGTAAAGATGCAGTATTG 3' and 5' TTGGAAATTTGGTGTTGCTGGTG 3'. Downstream genomic sequence was PCR-amplified using 5' AATCAAAGTGGTGCGAATAGAAATAC 3' and 5' AATGATGATAGTAAAACTGATGGTAGAAG 3'. Genomic sequences were cloned on either side of the Bsr cassette in pSP27-BSR (Wang *et al*., 2002) using XhoI/HindIII (5') and EcoRI (3'), generating the plasmid pSP72-BSR-EpsinKO. 10μg of linearized vector was transformed into Ax2 cells by electroporation. Cells

were diluted into 96-well plates and grown under Bsr selection. Clonal transformants lacking the entire *epnA* gene were identified by western blot and PCR analysis, and two of these clones, 10G10 and 5B4, were selected for further study.

cDNA cloning and sequence analysis

The protein sequence of human epsin1 (accession number NP037465) was used to search the *Dictyostelium* genome database (http://www.dictybase.org) for the best match using BLAST. A single homologous gene product was identified (DDB0183945, accession number XM630177). A complete cDNA clone was obtained from a *Dictyostelium discoideum* cDNA library using polymerase chain reaction (PCR) with primers 5' TGGAGACTATGATTAAAAGTTATATTAAAAAAGGTAAAGATGCAGTATTG AATACACCAGAAATTGAAAGAAAGGTTAG 3' and 5' GCAGATCCCATGCTATTAGTATTTCTATTCGC 3' and cloned into pCR2.1 using TA Cloning Kit (Invitrogen, Inc., Carlsbad, CA) to generate pCR2.1-Epsin. DNA sequences were managed using EditSeq and SeqMan (DNAStar, Inc., Madison, WI).

Cloning of expression plasmids pTX-epsin-GFP, pTX-epsin$_{1-333}$-GFP, pTX-GFP-epsin$_{253-677}$, pTX-epsin$_{253-677}$, pTX-GFP-PH, and pTX-PH:epsin$_{253-677}$

Epsin was cloned into pTX-GFP ((Levi *et al*., 2000), kind gift of T. Egelhoff) to generate pTx-EpsinGFP using KpnI and EcoRV. The cDNA encoding the epsin$_{1-333}$ truncation was PCR-amplified from pCR2.1-Epsin with 5' TGGAGACTATGATTAAAAGTTATATTAAAAAAGGTAAAGATGCAGTATTG AATACACCAGAAATTGAAAGAAAGGTTAG 3' and 5' GGTCGACTTCTTCCGCCAG 3' and ligated into pCR2.1 to generate pCR2.1-

epsin$_{1-333}$. pCR2.1-epsin$_{1-333}$ was then cut with EcoRI, blunted, and cloned into the EcoRV site of pTxGFP, making pTX- pCR2.1-epsin$_{1-333}$GFP. Epsin$_{253-677}$ was amplified by PCR from pCR2.1-Epsin using 5' TATAGTAATAGAGCAGGTGAGGAAACAAGAAG 3' and 5' CAGATCCCATGCTATTAGTATTTCTATTCGC 3' and ligated into pCR2.1 to make pCR2.1- epsin$_{253-677}$. Epsin$_{253-677}$ was cloned from pCR2.1- epsin$_{253-677}$. into pTx-GFP using BamHI and XhoI, making the expression plasmid pTx-GFP- epsin$_{253-677}$.. A cDNA encoding the PH domain of PLCδ (kind gift of T. Meyer) was cloned into pTxGFP using BamHI and NotI to generate pTx-GFP-PH. PH:epsin$_{253-677}$ was generated by cloning the PH domain into pCR2.1-epsin$_{253-677}$ with HindIII and SacIto make pCR2.1- PH:epsin$_{253-677}$. PH:epsin$_{253-677}$ was then cloned into pUC18 (Invitrogen, Inc., Carlsbad, CA) with HindIII and HincII to make pUC18- PH:epsin$_{253-677}$. Finally, PH:epsin$_{253-677}$ was cloned into pTx-GFP with KpnI, making pTX- PH:epsin$_{253-677}$GFP. To generate pTX-epsin$_{253-677}$, an expression plasmid for epsin$_{253-677}$ without the GFP tag, we amplified a cDNA encoding epsin$_{253-677}$ with 5' CAGTGTGCTGGTACCCGGCTTTATAGTAATAG 3' and 5' GATGGATAGGATCCTAATTCGGCTTCAG 3', ligated into pCR2.1, and cloned into pTxGFP with KpnI and BamHI, effectively replacing GFP with epsin$_{253-677}$. Plasmid maps were managed using Gene Construction Kit (Textco BioSoftware, West Lebanon, NH).

Dictyostelium transformation

Dictyostelium cell lines were transformed with various expression plasmids by electroporation. 5x10^6 cells in 100 μl of buffer H-50 (20mM HEPES, 50mM KCl, 10mM NaCl, 1mM $MgSO_4$, 5mM $NaHCO_3$, 1mM NaH_2PO_4) were mixed with 10 μg

of plasmid and electroporated using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA, USA) at 75 kV and 25 μF.

Fluorescence microscopy

Cells expressing GFP expression plasmids were harvested and allowed to attach to glass coverslips for 10 min at 18°C and incubated with low fluorescence media (Liu *et al.*, 2002) for at least 20min. Cells were fixed with 2% Formaldehyde and 0.01% Triton-X 100 in PDF (2 mM KCL, 1.1 mM K_2HPO_4, 1.32 mM KH_2PO_4, 0.1 mM $CaCL_2$, 0.25 mM $MgSO_4$, pH 6.7) at room temperature for 15min and then in 100% methanol at -20°C for 5min, then rinsed with PDF and mounted on glass slides. For immunostaining, cells on coverslips were blocked with 3% BSA (Fisher Scientific, Pittsburgh, PA) in PBS (137mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.4) for 20min at 37°C, and then incubated with rabbit anti-CLC (Wang *et al.*, 2003) or rabbit anti-AP2 IgG for 1hr. Coverslips were rinsed with PBS, incubated 30min with 30μg/mL goat anti-rabbit IgG conjugated to Texas Red (Molecular Probes, Invitrogen, Inc., Carlsbad, CA), and rinsed again in PBS. Coverslips were then rinsed in sterile, distilled water and mounted on glass slides. Images were taken using an inverted Nikon Eclipse TE200 microscope (Nikon Instruments, Dallas, TX) with 100X 1.4 NA PlanFlour objective and a Quantix 57 camera (Roper Scientific, AZ) controlled by Metamorph software (Universal Images, PA). Confocal images were acquired on a Leica TCS-SP2 laser scanning confocal inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany). Images were processed using Metamorph (Molecular Devices, Sunnyvale, CA) and Adobe Photoshop (Adobe Systems, Inc, San Jose, CA) software.

Generation of anti-epsin antibodies

A cDNA for epsin$_{253-677}$ was cloned from pCR2.1- epsin$_{253-677}$ into pMAL-C2X (New England Biolabs, Inc, Ipswich, MA) with BamHI and PstI so that epsin$_{253-677}$ was downstream of maltose-binding protein (MBP), resulting in expression of MBP-epsin$_{253-677}$ fusion protein from the plasmid pMAL-MBP-epsin$_{253-677}$. The pMAL-MBP-epsin$_{253-677}$ expression plasmid was transformed into *Eschericha coli* BL21 and the fusion protein was purified according to manufacturer's protocol. Purified MBP-epsin$_{253-677}$ was used to generate anti-epsin polyclonal antisera in rabbits (Cocalico Biologicals, Reamstown, PA).

Osmoregulation

Cells were harvested and allowed to attach to glass coverslips for 10 in at 18°C. Cells were then shifted to sterile, distilled water and imaged on an inverted Nikon Eclipse TE200 microscope using DIC (differential interference contrast) optics.

Development and spores

To develop fruiting bodies, approximately 5x10^7 cells were harvested, washed with PDF, and plated on starvation agar plates (0.2mM $CaCl_2$, 2.0Mm $MgSO_4$, 20mM MES pH6.7, 1% Agar Noble (BD, Sparks, MD)). Fruiting bodies were allowed to develop for 48hrs and then imaged using a Zeiss STEMI SR stereoscope (Carl Zeiss, Inc, Thornwood, NY). Spores were harvested from development plates by sharply striking the inverted plate on a hard surface and resuspending spores from the lid in

PDF. Spores were plated on glass coverslips, allowed to settle, and imaged using an inverted Nikon Eclipse TE200 microscope with DIC optics.

Cytokinesis and growth in suspension

Cells were diluted to $1x10^{4-5}$ cells/mL and grown in HL-5 on a rotary shaker at 218r.p.m at 18°C. Cultures were sampled periodically and counted on a Bright Line improved Neubauer hemacytometer (Hausser Scientific, Hersham, PA). For DAPI (4',6-diamidino-2-phenylindole) staining, cells were grown in suspension for 72 hrs and then allowed to attach to glass coverslips for 10 minutes. Cells were fixed with 100% methanol at -20°C for 5 min and rinsed with PDF. Cells were then stained with 0.05μg/mL DAPI (Invitrogen, Inc, Carslbad, CA) in PDF for 10min, rinsed, mounted on glass slides, and imaged on an inverted Nikon Eclipse TE200 microscope.

Subcellular Fractionation

Cells were fractionated according to Wang *et al*. (2003). Briefly, cells were collected, washed, and resuspended to $4x10^7$ cells/ml in MES isolation buffer (10mM MES (pH 6.5), 50mM KAc, 0.5mM $MgCl_2$, 1mM EGTA, 1mM DTT, and 0.02% NaN_3) with protease inhibitors (Fungal Protease Inhibitor cocktail, Sigma-Aldrich, St. Louis, MO). Cells were lysed by passing through two pieces of Osmonics (GE Osmonics, Trevose, PA) polycarbonate membrane (pore size: 5 μm) in a Gelman Luer-Lock-style filter (Gelman Sciences, Ann Arbor, MI). Cell lysates were centrifuged at 3000x*g* for 10 min at 4°C to generate a low speed pellet (LSP) and low speed supernatant (LSS). The LSS was ultracentrifuged at 100,000x*g* for 60 min at 4°C, resulting in a high speed supernatant (HSS) and a high speed pellet (HSP).

Site-Directed Mutagenesis

Epsin/ENTH$^{R65A/K78A}$ and Epsin/ENTH^{T107A} were generated using the Stratagene Quick Change Site-Directed Mutagensis Kit (Stratagene, La Jolla, CA) with primer pairs 5'-AATTATTATGGGTGTAATTTGGAAAGCTATTAATGATCCAGGCAAGTTTTGG-3' and 5'-CCAAAACTTGCCTGGATCATTAATAGCTTTCCAAATTACACCCATAATAATT-3' (for R65A), 5'-GATCCAGGCAAGTTTTGGAGACATGTTTATGCATCACTTCTTCTTATCG-3' and 5'-CGATAAGAAGAAGTGATGCATAAACATGTCTCCAAAACTTGCCTGGATC-3' (for K78A), and 5'-GATTGTAGACATCATACTATGGAAATTAAAGCATTGGTTGAGTTCCAA-3' and 5'-TTGGAACTCAACCAATGCTTTAATTTCCATAGTATGATGTCTACAATC-3' (for T107A).

MBP:Epsin Binding Assay

100ml of *Dictyostelium* suspension culture was harvested, washed in ice-cold binding buffer (20 mM piperazine-N,N'-bis[2-ethanesulfonic acid], pH 6.8, 1.5 mM EDTA, 15 mM $MgCl_2$, 1 mM DTT, and fungal protease inhibitor cocktail) (Vithalani et al., 1998) and resuspended to a concentration of $5x10^7$ cells/mL. Cells were sonicated 5x15sec at 50% power and centrifuged at 14,000xg for 20 min. MBP-

epsin$_{253\text{-}677}$ or MBP alone was purified from 1L bacterial culture according to manufacturer's protocol (see above), with the exception that the protein was not eluted from the amylose resin. 400ul of beads were incubated with 1mL prepared *Dictyostelium* lysate for 2 hrs at 4°C with shaking. Beads were washed several times with cold binding buffer, and the bound fraction was eluted with hot RSB (*r*educing *s*ample *b*uffer). Samples were analyzed using standard immunoblotting protocols.

Lipid Binding Assay

1 nmol of PI or PI(4,5)P_2 (Echelon Biosciences, Inc, Salt Lake City, UT) was pipetted onto nitrocellulose membrane and allowed to air dry. Membrane was then blocked in ice-cold binding buffer with 3% dry milk for 30 minutes. *Dictyostelium* lysate was prepared as above and incubated with the membrane for 2hrs at 4°C with gentle shaking. Membrane was washed with 0.1% Tween-20 in PDF and then probed using standard immunoblotting protocols.

Cloning clathrinRFP

Clathrin light chain was cloned from pTxGFP:CLC (Wang et al., 2006b) into p333-9 mRFPmars BsrH expression vector (kind gift of Annette Muller-Taubenberger) with EcoRI and XhoI.

Triple Stain Microscopy

EpsinGFP and clathrinRFP were co-transformed into *Dictyostelium* as described above, using 10μg of each plasmid per transformation. Cell were then fixed for immunofluorescence as described above, using Pacific Blue-conjugated goat α-

rabbit IgG secondary antibody (Molecular Probes, Invitrogen, Inc., Carlsbad, CA). Cells were imaged on an inverted Nikon Eclipse TE200 microscope as described above, using FITC, rhodamine, and Pacific Blue filter sets (Chroma Technology Corp., Rockingham, VT).

Quantification of colocalization

Images were cropped and prepared for analysis using Adobe Photoshop (Adobe Systems, Inc, San Jose, CA) and ImageJ (U. S. National Institutes of Health, Bethesda, MD). Colocalization was measured using an ImageJ macro that scored each pixel for whether its intensity in each channel was above the mean image intensity for that channel. Code for this macro is in Appendix B.

Live cell imaging by TIRF Microscopy

Cells were imaged by TIRF (*t*otal *i*nternal *r*eflection *f*luorescence) microscopy at the University of Texas Southwestern Medical Center Imaging Core Facility with the assistance of Kate Luby-Phelps. Images were acquired on a Zeiss AxioObserver microscope using 488nm and 561nm lasers with a Roper QuantEM camera controlled by Slidebook software (Intelligent Imaging Innovations, Inc., Denver, CO).

Appendix B: ImageJ Macros

Macro B.1 Quantification of colocalization from triple-stain assays

```
//This macro calculates the mean for each color in an RGB image
//above a low baseline, then tallies the colocalization for each
//pixel and prints those results to the "results" window.

requires("1.35b");

//definition of variables

//"counter" and "sum" are used to calculate "mean" of whole image
//only pixels above a certain low baseline are counted in the mean
//to avoid skewing the results based on variable amounts of empty
//space in a given field of cells.

redCounter = 0;
redSum = 0;
meanRed = 0;

greenCounter = 0;
meanGreen = 0;
greenSum = 0;

blueCounter = 0;
meanBlue = 0;
blueSum = 0;

//"total" holds the tallies the total pixels above the mean for that channel

totalRed = 0;
totalGreen = 0;
totalBlue = 0;

//"only" holsd the tallies the number of pixels for that channel that have
//signal above the mean for only that channel
onlyRed = 0;
onlyGreen = 0;
onlyBlue = 0;
```

```
//these hold the talies for the pixels with signal above mean in the indicated channel

redGreen = 0;
redBlue = 0;
greenBlue = 0;
redGreenBlue = 0;

if (bitDepth!=24)
	exit("This macro requires an RGB image");

//gets the width and height of the image
width = getWidth();
height = getHeight();


//defines the baseline for each channel. Only pixels
//with intensities above the baseline will be included
//in the mean

baselineRed =10;
baselineGreen = 10;
baselineBlue =10;

//loops through each pixel to calculate the image mean for each channel
for(i=0; i<width; i++)
	for(j=0; j<height; j++)
	{
		v = getPixel( i, j); //get pixel value for current pixel
		red = (v>>16)&0xff;  // extract red byte (bits 23-17)
	green = (v>>8)&0xff; // extract green byte (bits 15-8)
	blue = v&0xff;       // extract blue byte (bits 7-0)

		if (red>baselineRed || green>baselineGreen || blue>baselineBlue)
		{
			if(red>baselineRed)
			{
				redSum = redSum + red;
				redCounter++;
			}

			if(green>baselineGreen)
			{
```

```
                        greenSum = greenSum + green;
                        greenCounter++;
                }

                if(blue>baselineBlue)
                {
                        blueSum = blueSum + blue;
                        blueCounter++;
                }
        }// end big if statement
} //end inner for loop

meanRed = round(redSum/redCounter);
meanGreen = round(greenSum/greenCounter);
meanBlue = round(blueSum/blueCounter);

//loops through the image again, this time to tally colocalization
for(i=0; i<width; i++)
        for(j=0; j<height; j++)
        {
                v = getPixel( i, j); //get pixel value for current pixel
                red = (v>>16)&0xff;  // extract red byte (bits 23-17)
        green = (v>>8)&0xff; // extract green byte (bits 15-8)
        blue = v&0xff;       // extract blue byte (bits 7-0)

                if (red>meanRed || green>meanGreen || blue>meanBlue)
                {
                if(red > meanRed)
                        totalRed++;
                if(green > meanGreen)
                        totalGreen++;
                if(blue > meanBlue)
                        totalBlue++;

                if(red > meanRed && green <= meanGreen && blue <= meanBlue)
                        onlyRed++;
                if(red <= meanRed && green > meanGreen && blue <= meanBlue)
                        onlyGreen++;
                if(red <= meanRed && green <= meanGreen && blue > meanBlue)
                        onlyBlue++;

                if(red > meanRed && green > meanGreen && blue <= meanBlue)
                        redGreen++;
```

```
                if(red > meanRed && green <= meanGreen && blue > meanBlue)
                        redBlue++;
                if(red <= meanRed && green > meanGreen && blue > meanBlue)
                        greenBlue++;
                if(red > meanRed && green > meanGreen && blue > meanBlue)
                        redGreenBlue++;
        }// end big if statement
        } //end inner for loop

//opens the "results" window and measures any options selected in the "measure" tab
run("Measure");

//sends results to the "results" table, with co-loc as a percentage

setResult("Red baseline", nResults-1, baselineRed);
setResult("Green baseline", nResults-1, baselineGreen);
setResult("Blue baseline", nResults-1, baselineBlue);
setResult("Red mean", nResults-1, meanRed);
setResult("Green mean", nResults-1, meanGreen);
setResult("Blue mean", nResults-1, meanBlue);

setResult("red and green", nResults-1, (redGreen/totalRed));
setResult("red and blue", nResults-1, (redBlue/totalRed));
setResult( "all three (red)", nResults-1, (redGreenBlue/totalRed));
setResult("Red only", nResults-1, (onlyRed/totalRed));

setResult("Green and red", nResults-1, (redGreen/totalGreen));
setResult("Green and blue", nResults-1, (greenBlue/totalGreen));
setResult( "all three (green)", nResults-1, (redGreenBlue/totalGreen));
setResult("Green only", nResults-1, (onlyGreen/totalGreen));

setResult("Blue and green", nResults-1, (greenBlue/totalBlue));
setResult("Blue and red", nResults-1, (redBlue/totalBlue));
setResult( "all three (blue)", nResults-1, (redGreenBlue/totalBlue));
setResult("Blue only", nResults-1, (onlyBlue/totalBlue));

//necessary for results to print to the results table
updateResults();

//closes the image
close();
```

Macro B.2 Quantification of colocalization from double-stain assays

```
//this macro calculates the mean for only the red and green color in an
//RGB image above a low baseline, then calculates the percentage
//colocalization for each pixel above the mean in each color and prints
//those results to the "results" window. The logic and purpose of the
//variables and calculations is the same as the Macro B.1


requires("1.35b");

redCounter = 0;
redSum = 0;
meanRed = 0;

greenCounter = 0;
meanGreen = 0;
greenSum = 0;

totalRed = 0;
totalGreen = 0;

onlyRed = 0;
onlyGreen = 0;
redGreen = 0;


  if (bitDepth!=24)
    exit("This macro requires an RGB image");

width = getWidth();
height = getHeight();


//this gets the mean of each color, counting only pixels
//above a baseline value

baselineRed =10;
baselineGreen = 10;

for(i=0; i<width; i++)
          for(j=0; j<height; j++)
```

```
	{
		v = getPixel( i, j); //get pixel value for current pixel
		red = (v>>16)&0xff;  // extract red byte (bits 23-17)
	green = (v>>8)&0xff; // extract green byte (bits 15-8)
	blue = v&0xff;		// extract blue byte (bits 7-0)

		if (red>baselineRed || green>baselineGreen)
		{
			if(red>baselineRed)
			{
				redSum = redSum + red;
				redCounter++;
			}

			if(green>baselineGreen)
			{
				greenSum = greenSum + green;
				greenCounter++;
			}


		}// end big if statement
	} //end inner for loop

meanRed = round(redSum/redCounter);
meanGreen = round(greenSum/greenCounter);


//this scans the image again, this time to count colocalization

for(i=0; i<width; i++)
	for(j=0; j<height; j++)
	{
		v = getPixel( i, j); //get pixel value for current pixel
		red = (v>>16)&0xff;  // extract red byte (bits 23-17)
	green = (v>>8)&0xff; // extract green byte (bits 15-8)
	blue = v&0xff;		// extract blue byte (bits 7-0)

		if (red>meanRed || green>meanGreen)
		{
			if(red>meanRed)
				totalRed++;
			if(green>meanGreen)
```

```
                totalGreen++;

            if(red>meanRed && green<=meanGreen)
                onlyRed++;
            if(red<=meanRed && green>meanGreen)
                onlyGreen++;
            if(red>meanRed && green>meanGreen)
                redGreen++;

        }// end big if statement
    } //end inner for loop

run("Measure");

setResult("Red baseline", nResults-1, baselineRed);
setResult("Green baseline", nResults-1, baselineGreen);

setResult("Red mean", nResults-1, meanRed);
setResult("Green mean", nResults-1, meanGreen);

setResult("Red only", nResults-1, (onlyRed/totalRed));
setResult("red and green", nResults-1, (redGreen/totalRed));

setResult("Green only", nResults-1, (onlyGreen/totalGreen));
setResult("Green and red", nResults-1, (redGreen/totalGreen));

updateResults();
close();
```

Macro B.3 Quantification of intensity of a puncta during time-lapse microscopy: single channel version

```
//This macro measures the mean intensity of pixels in a selection
//and normalizes by subtracting out the mean of the whole cell
//results for each slice are printed to the "Results" window

requires('1.34h');

//you must start by inputting an arbitrary height and center point
//to draw a circle around the puncta:x and y are the center of the
//selection
```

```
x =110;
y = 129;

//the radius of the circle drawn around the indicated puncta
radius = 4;

//first and last slice of the portion of the time-lapse
//stack you want to analyze
minSlice = 1;
maxSlice = 120;

//number of slices in the stack
slices = nSlices;

//avoids an error message if you entered a maxSlice value
//greater than teh total number of slices
if(maxSlice > slices)
	maxSlice = slices;

//if you wish to scale the calculated background value
scaleFactor = 1;

//draws a new circle around indicated center
makeOval((x-radius), (y-radius), (radius*2), (radius*2));

//loops through each slice to measure the circle on each slice
for(n=minSlice; n<=maxSlice; n++)
{
	setSlice(n);

	//resets the variables for the loop
	baselineSum = 0;
	imageSum = 0;
	counter = 0;

	//calculates the baseline average based on a
	//10x10 square in upper left corner of slice

	for(i=0; i<10; i++)
		for(j=0; j<10; j++)
		{
			pixelValue=getPixel(i,j);
```

```
                baselineSum += pixelValue;
        } //end for loops

    baseline = baselineSum/100;

    //calculates the image mean above the baseline*scaleFactor
    imageWidth = getWidth();
    imageHeight = getHeight();

    for(i=0; i<imageWidth; i++)
        for(j=0; j<imageHeight; j++)
        {
            pixelValue=getPixel(i,j);
            if (pixelValue>(baseline*scaleFactor))
            {
                imageSum += pixelValue;
                counter++;
            } //end if statement
        } //end for loops

    imageMean = imageSum/counter;

    //this gets the mean of the selection. "mean" must be selected
    //in the "measure" tab
    run("Measure");
    if (isNaN(mean))
        exit("Mean must be be selected in Analyze>Set Measurements");

    mean=getResult('Mean', nResults-1);
    setResult("Baseline", nResults-1, baseline);
    setResult("ImageMean", nResults-1, imageMean);
    setResult("Signal above Mean", nResults-1, mean-imageMean);
    setResult("CenterX", nResults-1, x);
    setResult("centerY", nResults-1, y);

    updateResults();

} //end big outer for loop
```

Macro B.4 Quantification of intensity of a puncta during time-lapse microscopy: Red and Green version

```
//this macro measures the mean intensity of a selection in red and green channels and
//prints results to a separate table. Headings are labeled for clathrin and LimE
//measurement. Algorithm is similar to Macro B.3

requires('1.34h');
requires("1.35b");


x =130;
y = 116;
radius = 4;
minSlice = 1;
maxSlice = 200;
slices = nSlices;
if (maxSlice > slices)
        maxSlice = slices;

//title of the new results window
title = "2-27-08 AX2 LimE CLC 1-2-1";
title2 = "["+title+"]";

//creates a table and prints the headings
run("New... ", "name="+title2+" type=Table width=250 height=600");
print(title2, "\\Headings:slice\tx\ty\tr\tLimE baseline\tLimE image mean\tLimE
selection mean\tLimE signal above mean\tCLC baseline\tCLC image mean\tCLC
selection mean\tCLC signal above mean");

redScaleFactor = 1;
greenScaleFactor = 1;

redFluorophore = "LimE TIRF";
greenFluorophore = "CLC TIRF";

//draws a new circle around indicated center
makeOval((x-radius), (y-radius), (radius*2), (radius*2));

//loops through stack to measure the circle on each slice
for(n=minSlice; n<=maxSlice; n++)
{
        setSlice(n);
```

```
//resets the variables for the loop
redBaselineSum = 0;
redImageSum = 0;

greenBaselineSum = 0;
greenImageSum = 0;

redCounter = 0;
greenCounter = 0;

//calculates the baseline average based on a
//10x10 square in upper left corner of image

for(i=0; i<10; i++)
        for(j=0; j<10; j++)
        {
                v = getPixel( i, j); //get pixel value for current pixel
                red = (v>>16)&0xff;  // extract red byte (bits 23-17)
                green = (v>>8)&0xff; // extract green byte (bits 15-8)
                blue = v&0xff;       // extract blue byte (bits 7-0)

                redBaselineSum += red;
                greenBaselineSum += green;
        } //end for loops

redBaseline = redBaselineSum/100;
greenBaseline = greenBaselineSum/100;

//calculates the image mean above the baseline for each color
imageWidth = getWidth();
imageHeight = getHeight();

for(i=0; i<imageWidth; i++)
        for(j=0; j<imageHeight; j++)
        {
                v = getPixel( i, j); //get pixel value for current pixel
                red = (v>>16)&0xff;  // extract red byte (bits 23-17)
                green = (v>>8)&0xff; // extract green byte (bits 15-8)
                blue = v&0xff;       // extract blue byte (bits 7-0)

                if (red>(redBaseline*redScaleFactor))
                {
```

```
                        redImageSum += red;
                        redCounter++;
                    } //end if statement

                    if (green>(redBaseline*greenScaleFactor))
                    {
                        greenImageSum += green;
                        greenCounter++;
                    } //end if statement
                } //end for loop

        redImageMean = redImageSum/redCounter;
        greenImageMean = greenImageSum/greenCounter;

        //this gets the mean of the red selection
        setRGBWeights(1, 0, 0);
        run("Measure");
        redSelectionMean=getResult('Mean', nResults-1);
        redSignalAboveMean = redSelectionMean - redImageMean;

        //this gets the mean of the green selection
        setRGBWeights(0, 1, 0);
        run("Measure");
        greenSelectionMean=getResult('Mean', nResults-1);
        greenSignalAboveMean = greenSelectionMean - greenImageMean;

        //prints the calculations to the new table
        print(title2,n+"\t"+x+"\t"+y+"\t"+radius+"\t"+redBaseline+"\t"+redImageMea
n+"\t"+redSelectionMean+"\t"+redSignalAboveMean+"\t"+greenBaseline+"\t"+green
ImageMean+"\t"+greenSelectionMean+"\t"+greenSignalAboveMean);

} //end big outer for loop
```

Appendix C: Cell lines used in this study

cell line	null mutation	parent line	selectable marker	
AX2	wild-type	-	-	
5B4	epsin	AX2 (WT)	Bsr	
10G10	epsin	AX2 (WT)	Bsr	
6A5	α–adaptin	AX2 (WT)	Bsr	Yujia Wen
4F6	Hip1r	AX2 (WT)	Bsr	(Repass et al., 2007)
5H11	AP180	AX2 (WT)	Bsr	(Stavrou and O'Halloran, 2006)
2A1	clathrin light chain	NC4A2 (WT)	Bsr	(Wang et al., 2003)
5E2	clathrin heavy chain	AX2 (WT)	Bsr	(Ruscetti et al., 1994)
DH1	wild-type	-	-	
E4B1	epsin	DH1 (WT)	Bsr	
3E1	α–adaptin	DH1 (WT)	Pyr 5/6	Yujia Wen
3D4	Hip1r	DH1 (WT)	Pyr 5/6	Shannon Repass
1A9	epsin and α–adaptin	3E1	Pyr 5/6 and Bsr	
3H11	epsin and Hip1r	3D4	Pyr 5/6 and Bsr	(Repass et al., 2007)

Appendix D: Miscellaneous Experiments

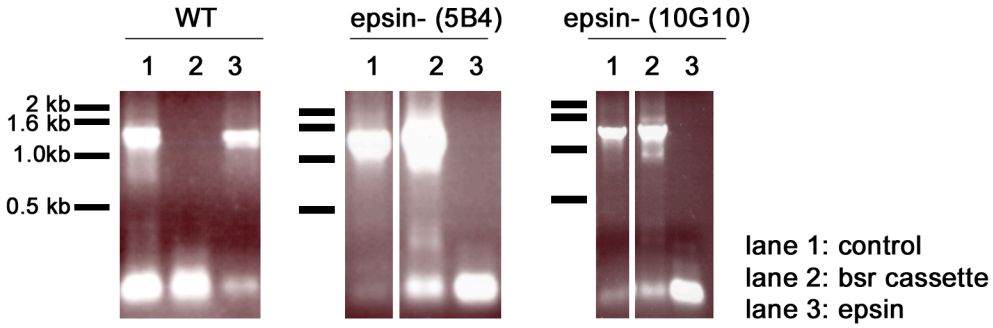

Figure D.1 Disruption of *epnA* confirmed by PCR

Preparations of genomic DNA from wild-type (**WT**) and two independent epsin null cell lines (**epsin- (5B4)** and **epsin- (10G10)**) were analyzed by PCR and run on a 1% agarose gel with 0.1mg/ml EtBr. Lane 1: PCR reaction using control primers. Lane 2: PCR reaction using a primer from upstream of the *epnA* locus and a primer from within the inserted *bsr* cassette. Lane 3: PCR reaction using primers from within the *epnA* locus.

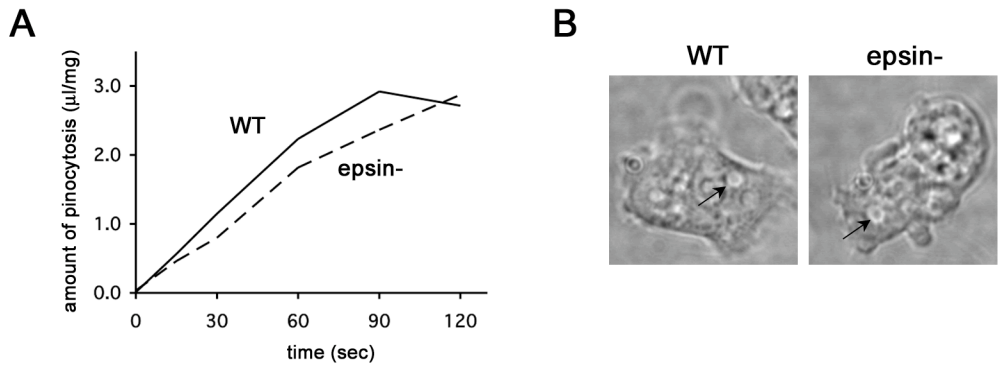

Figure D.2 Epsin null cells do not display defects in bulk fluid phase endocytosis or osmoregulation

(A) Epsin null cells undergo pinocytosis at levels similar to wild-type. Wild-type (**WT**) and epsin null cells (**epsin-**) were incubated with TRITC-dextran in shaking culture for 120 minutes. Samples were removed periodically and the amount of internalized dextran measured on a fluorimeter against a standard curve. Rates on pinocytosis were normalized against cellular protein (mg) as determined by Bradford assay. (B) Epsin null cells form normal contractive vacuoles. Wild-type (**WT**) and epsin null cells (**epsin-**) were incubated in hypoosmotic conditions for 60 minutes. Still frames from time-lapse microscopy with DIC optics are shown. Arrows indicate contractile vacuoles.

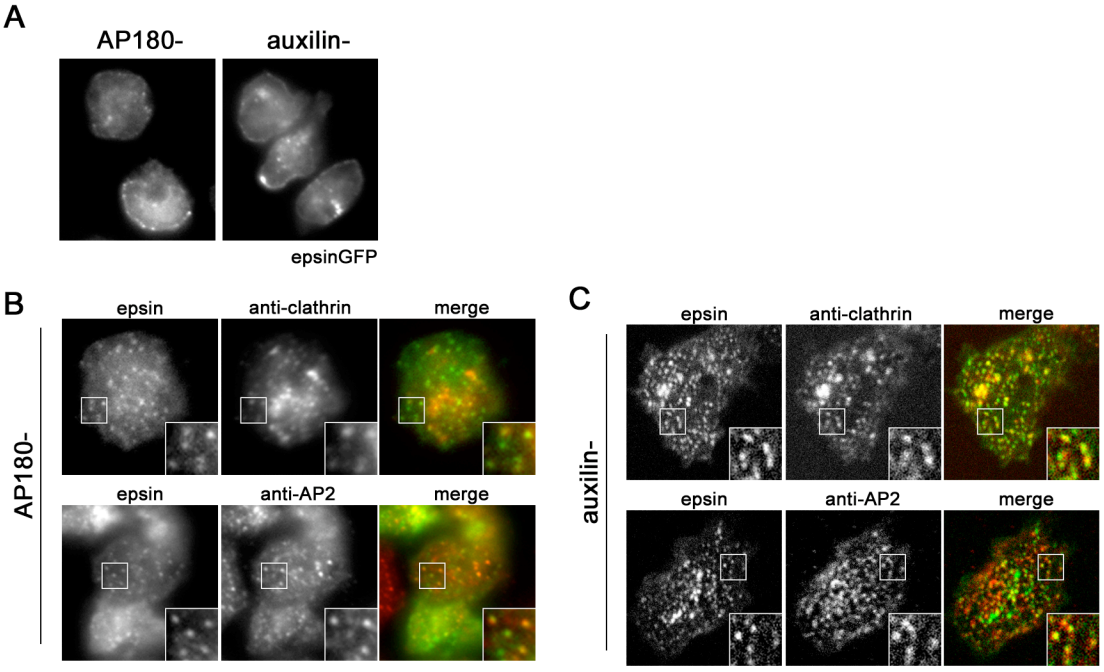

Figure D.3 Epsin colocalizes with clathrin and AP2 in auxilin null and AP180 null mutants

(A) AP180 null (**AP180-**, left) and auxilin null (**auxilin-**, right) cells expressing epsinGFP were fixed and examined under epifluorescence microscopy. Note how epsin forms membrane-associated puncta in both mutants, but epsinGFP tends to form aggregates at the membrane of auxilin null cells. (B) Epsin colocalizes with clathrin (top row) and AP2 (bottom row) in AP180 null cells. AP180 null cells expressing epsinGFP (epsin) were fixed and immunostained with anti-clathrin light chain (**anti-clathrin**) or anti-α-adaptin (**anti-AP2**) antibodies. (C) Epsin colocalizes with clathrin (top row) and AP2 (bottom row) in auxilin null cells. Auxilin null cells expressing epsinGFP (epsin) were fixed and immunostained with anti-clathrin light chain (**anti-clathrin**) or anti-α-adaptin (**anti-AP2**) antibodies.

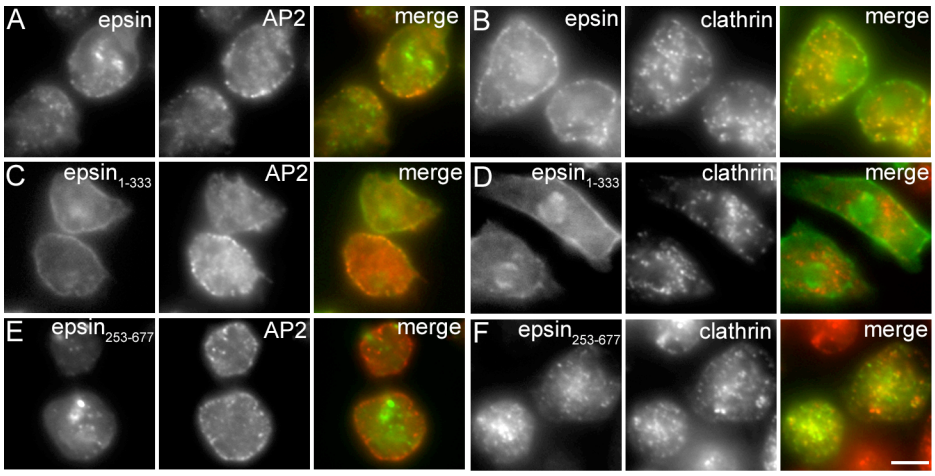

Figure D.4 The ENTH domain is required but not sufficient for epsin association with clathrin and AP2 at the plasma membrane of wild-type cells.

(A and B) EpsinGFP colocalizes with AP2 at the plasma membrane and clathrin at the plasma membrane and cytoplasm of wild-type cells. Wild-type cells expressing epsinGFP (green) were fixed and immunostained with anti-α-adaptin antibodies (A) or anti-clathrin antibodies (B) (red). (C and D) $Epsin_{1-333}GFP$ uniformly decorates the plasma membrane but does not form puncta. Wild-type cells expressing $epsin_{1-333}GFP$ (green) were fixed and immunostained with anti-α-adaptin antibodies (C) or anti-clathrin antibodies (D) (red). (E) $Epsin_{253-677}GFP$ does not form puncta at the plasma membrane and does not colocalize with AP2. Wild-type cells expressing $epsin_{253-677}GFP$ (green) were fixed and immunostained with anti-α–adaptin antibodies (red). (F) $Epsin_{253-677}GFP$ cytoplasmic puncta occasionally colocalize with clathrin. Wild-type cells expressing $epsin_{253-677}$:GFP (green) were fixed and immunostained with anti-clathrin antibodies (red). Bar, 5μm.

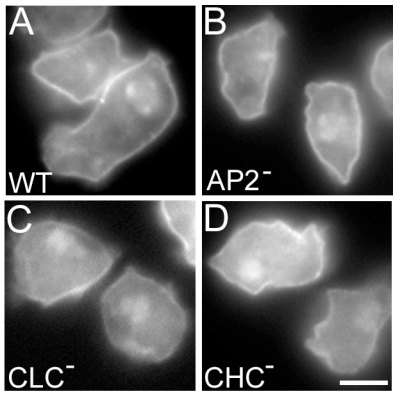

Figure D.5. The ENTH domain does not require clathrin or AP2 to localize to the plasma membrane.

(A) Wild-type (WT), (B) alpha-adaptin null ($AP2^-$), (C) clathrin light chain null (CLC^-), and (D) clathrin heavy chain null (CHC^-) cells expressing $epsin_{1-333}$:GFP were fixed and imaged with fluorescence microscopy. Bar, 5μm.

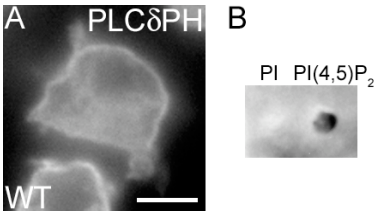

Figure D.6. PH domain of PLCδ binds $PI(4,5)P_2$ and localizes to the plasma membrane.

(A) Wild-type (WT) cells expressing PLCδPH:GFP. Bar, 5μm. (B) PI and $PI(4,5)P_2$ were pipetted onto nitrocellulose membrane and then incubated with lysate from *Dictyostelium* cells expressing PLCδPH:GFP. Blots were probed with anti-GFP antibody.

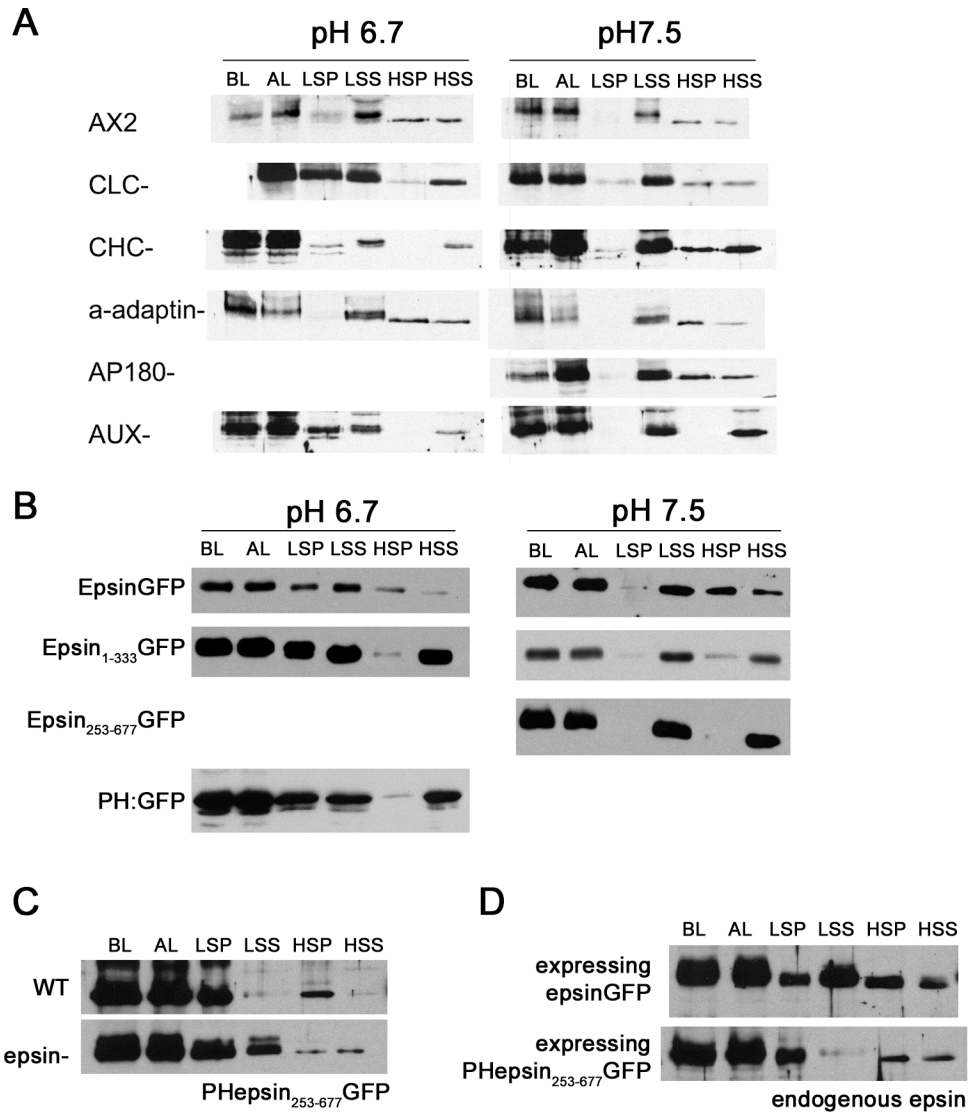

Figure D.7 Subcellular fraction of Epsin and epsin truncation in various conditions and backgrounds

Cells in two different buffer conditions (**pH 6.7** and **pH 7.5**) were lysed (**BL**, before lysis; **AL**, after lysis) centrifuged at 5,000xg for 5 min (**LSP**, low speed pellet; **LSS**, low speed supernatant). Supernatants were then centrifuged at 100,000xg for 1 hr (**HSP**, high speed pellet; **HSS** high speed supernatant). Samples were analyzed by immunoblot. (A) Epsin fractionation in wild-type (**AX2**), clathrin light chain null (**CLC-**), clathrin heavy chain null (**CHC-**), α–adaptin null (**α–adaptin-**), AP180 null (**AP180-**) and auxilin null (**AUX-**) cells. (B) $Epsin_{1-333}GFP$ associates more with membrane fractions and $epsin_{253-677}GFP$ associates more with soluble fractions as

(Figure D.7, continued) compared to full-length epsinGFP. Lysates of epsin null cells expressing epsinGFP, epsin$_{1\text{-}333}$GFP, epsin$_{253\text{-}677}$GFP, and PH:GFP were fractionated as described and analyzed by immunoblot with anti-GFP antibodies. (C) PHepsin$_{253\text{-}677}$GFP associates with slightly more soluble fractions in epsin null cells. Wild-type (**WT**) and epsin null (**epsin-**) cells expressing PHepsin$_{253\text{-}677}$GFP were fractionated and analyzed by immunoblot. (D) Expression of PHepsin$_{253\text{-}677}$GFP changes the fractionation pattern of endogenous epsin. Wild-type cells expressing either epsinGFP or PHepsin$_{253\text{-}677}$GFP were fractionated and analyzed by immunoblot probed with anti-epsin antibodies. Endogenous epsin band shown.

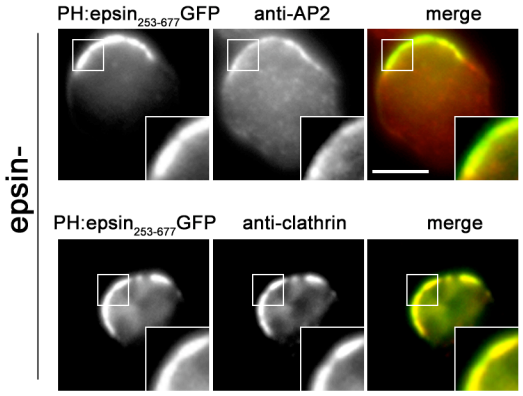

Figure D.8 PH:epsin$_{253\text{-}677}$GFP mislocalizes clathrin and AP2 in epsin null cells.

Epsin null (**epsin-**) cells expressing PH:epsin$_{253\text{-}677}$GFP were fixed and immunostained with anti-alpha adaptin (**anti-AP2**, top row) or anti-clathrin light chain (**anti-clathrin**, bottom row) antibodies and imaged under epifluorescence microscopy.

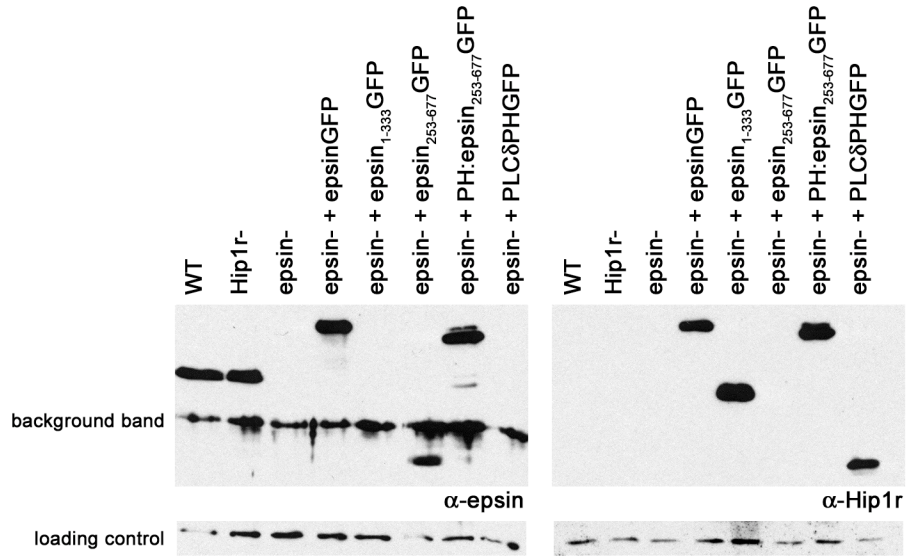

Figure D.9 Relative expression levels of constructs used in Chapter 2.

Lysates from wild-type (**WT**), Hip1r null (**Hip1r-**) and epsin null (**epsin-**) cell lines as well as epsin null cells expressing various GFP constructs were analyzed by immunoblot a probed with either anti-epsin (left) or anti-GFP (right) antibodies.

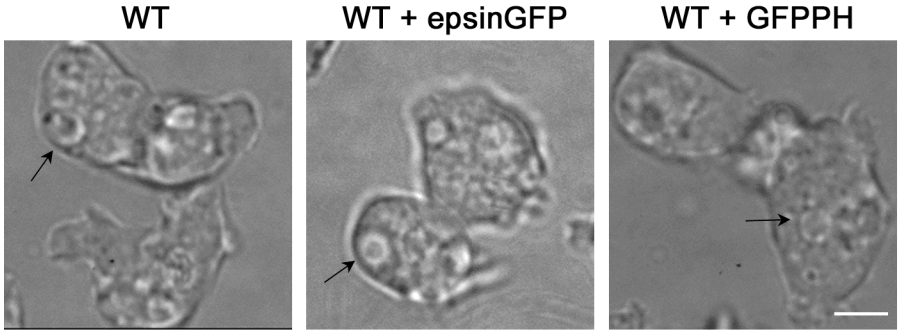

Figure D.10 Osmoregulation is not affected by the expression of epsinGFP or GFP:PH

Wild-type (**WT**) cells expressing epsinGFP (**WT + epsinGFP**) or GFP:PH (**WT + GFP:PH**) were placed in hypoosmotic conditions and imaged under DIC optics. Contractile vacuoles (arrows) of all cell lines are of comparable size. Bar, 5μm.

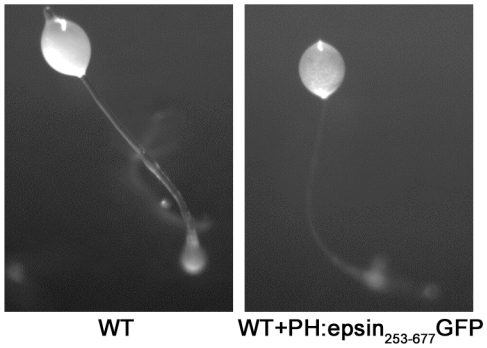

Figure D.11 Wild-type cells expressing PH:epsin$_{253\text{-}677}$GFP form normal fruiting bodies

Wild-type cells (**WT**, left) and wild-type cells expressing PH:epsin$_{253\text{-}677}$GFP (**WT + PH:epsin$_{253\text{-}677}$GFP**, right) were plated on agar plates and allowed to develop into fruiting bodies.

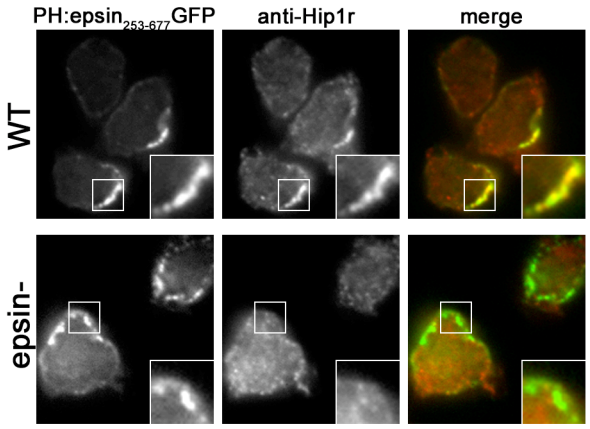

Figure D.12 PH:epsin$_{253\text{-}677}$GFP mislocalized Hip1r in wild-type but not epsin null cells.

Wild-type (**WT**, top row) or epsin null (**epsin-**, bottom row) cells expressing PH:epsin$_{253\text{-}677}$GFP were fixed and immunostained with anti-Hip1r antibodies. Note the presence of aggregated Hip1r at the membrane of wild-type, but not epsin null, cells.

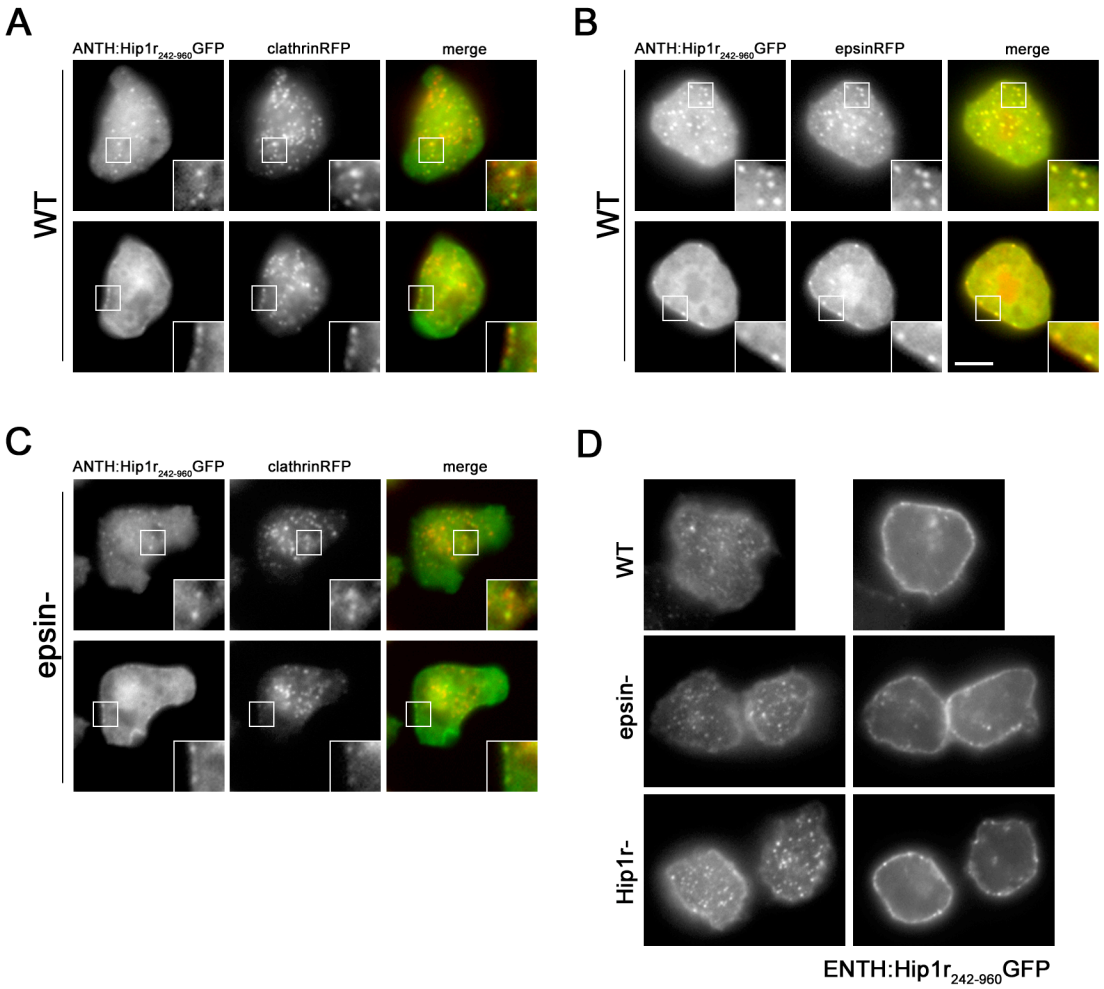

Figure D.13 Localization of ANTH:Hip1r$_{242-960}$GFP and ENTH:Hip1r$_{242-960}$GFP

(A) Wild-type (**WT**) cells co-expressing ANTH:Hip1r$_{242-960}$GFP and clathrin light chain:RFP (**clathrinRFP**) were fixed and imaged under epifluorescence microscopy. Two focal planes of the same cell are shown, one focused on the cell surface (top row) and one focused in the middle of the cell (bottom row). (B) Wild-type (**WT**) cells co-expressing ANTH:Hip1r$_{242-960}$GFP and epsinRFP were imaged as above. Two focal planes of the same cell are shown, one focused on the cell surface (top row) and one focused in the middle of the cell (bottom row). (C) Epsin null (**epsin-**) cells co-expressing ANTH:Hip1r$_{242-960}$GFP and clathrin light chain:RFP (**clathrinRFP**) were fixed and imaged as above. (D) Wild-type (**WT,** top row), epsin null (**epsin-**, middle row), and Hi1pr null (**Hip1r-**, bottom row) cells expressing ENTH:Hip1r$_{242-960}$GFP were fixed and imaged under epifluorescence microscopy. Two focal planes of the same field are shown, one focused on the cell surface (left) and one focused in the middle of the cell (right).

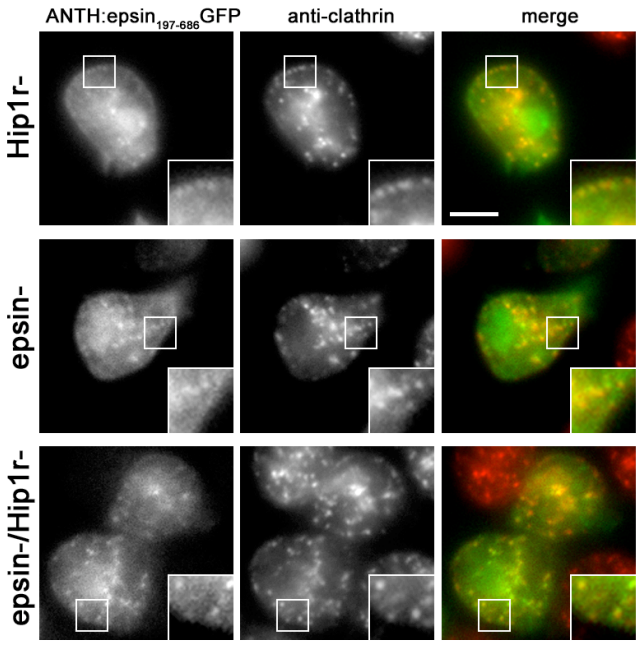

Figure D.14 Localization of ANTH:epsin$_{197-686}$GFP

Hip1r null (**Hip1r-**, top row), epsin null (**epsin-**, middle row), and epsin/Hip1r double null (**epsin-/Hip1r-**) cells expressing ANTH:epsin197-686GFP were fixed and immunostained with anti-clathrin light chain antibodies (**anti-clathrin**). Cells were imaged under epifluorescence microscopy. Bar, 5 μm.

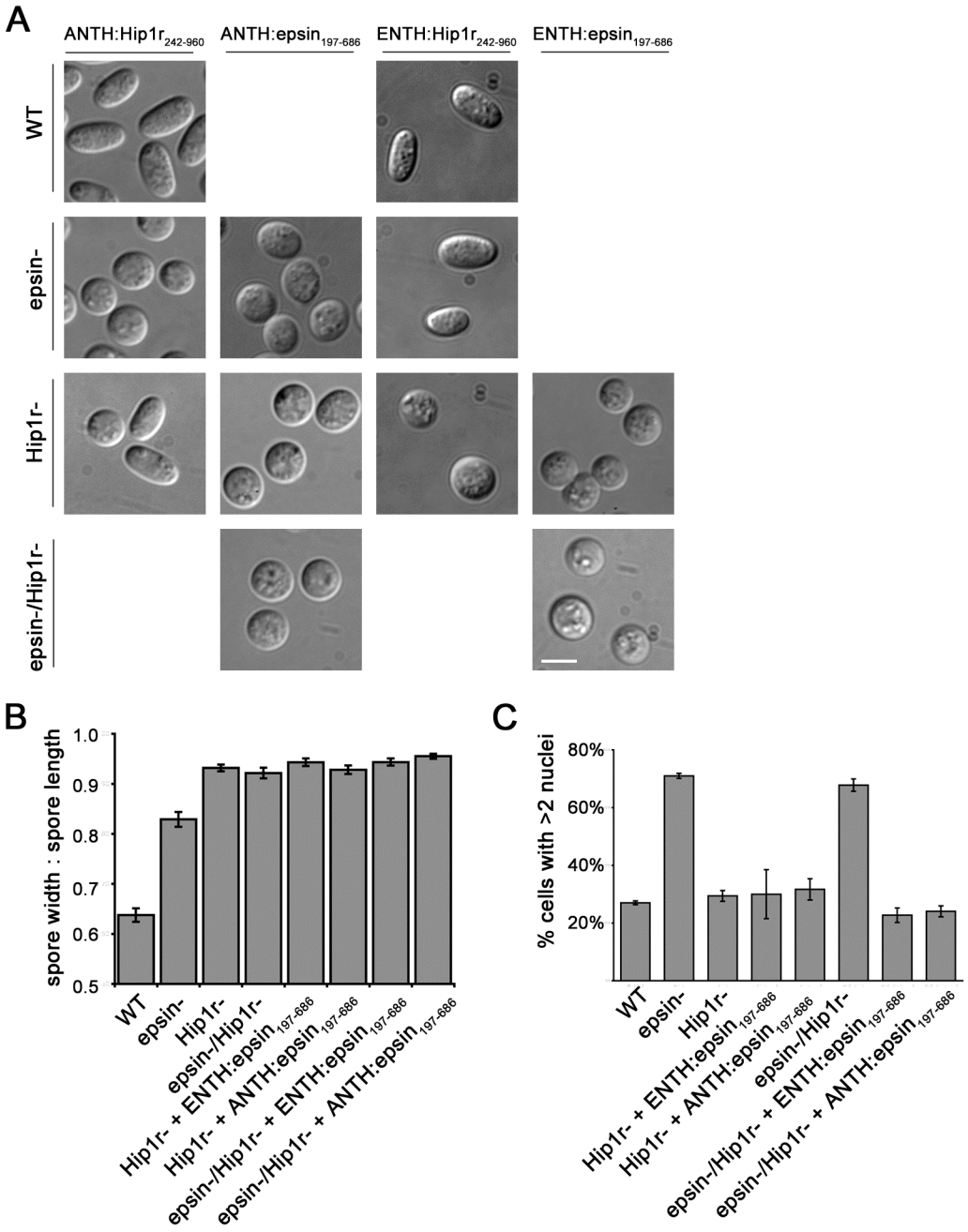

Figure D.15 Phenotypic rescue by epsin/Hip1r chimeric constructs

(Figure D.15, continued) (A) ANTH:Hip1r$_{242-960}$GFP partially rescues spore morphology in Hip1r null cells, while ENTH:Hip1r$_{242-960}$GFP fully rescues epsin null cells but not Hip1r null cells. Wild-type (**WT**), epsin null (**epsin-**), Hip1r null (**Hip1r-**) and epsin/Hip1r double null (**epsin-/Hip1r-**) cells expressing ANTH:Hip1r$_{242-960}$GFP, ENTH:epsin$_{197-686}$GFP, ANTH:epsin$_{197-686}$GFP, or ENTH:epsin$_{197-686}$GFP were allowed to develop under starvation on plates. Spores were harvested and imaged by DIC optics. Bar, 5μm. (B) Quantification of spore morphology. Spores from several of the above cell lines were measured and their width:length ratio calculated; n = 50 for each cell line, error bars are standard error. (C) Quantification of cytokinesis defects. Cells from several of the above cells lines were grown in suspension culture for 72 hrs, then stained with DAPI and imaged under epifluorescence microscopy. n = 3 trials of 100 cells each; error bars are standard error.

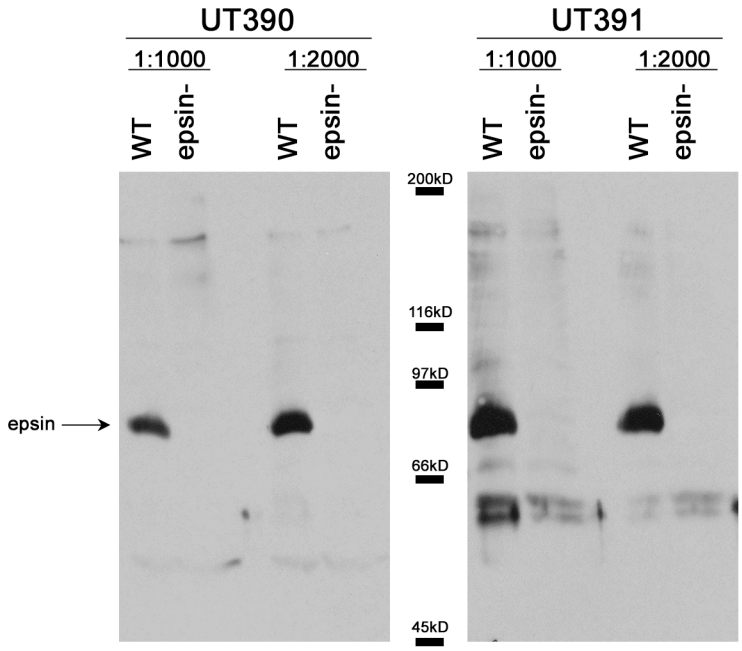

Figure D.16 Antibodies UT390 and UT391 are specific for epsin

Two rabbit antibodies, UT390 and UT391, were raised against the carboxy-terminal portion of *Dictyostelium* epsin. Antibody specificity was tested by immunoblot using lysates from wild-type (**WT**) and epsin null (**epsin-**) cell lines. Antibodies were tested at two concentrations, 1:1000 and 1:2000. These antibodies were also tested for immunofluorescence but did not give good signal, even after affinity purification.

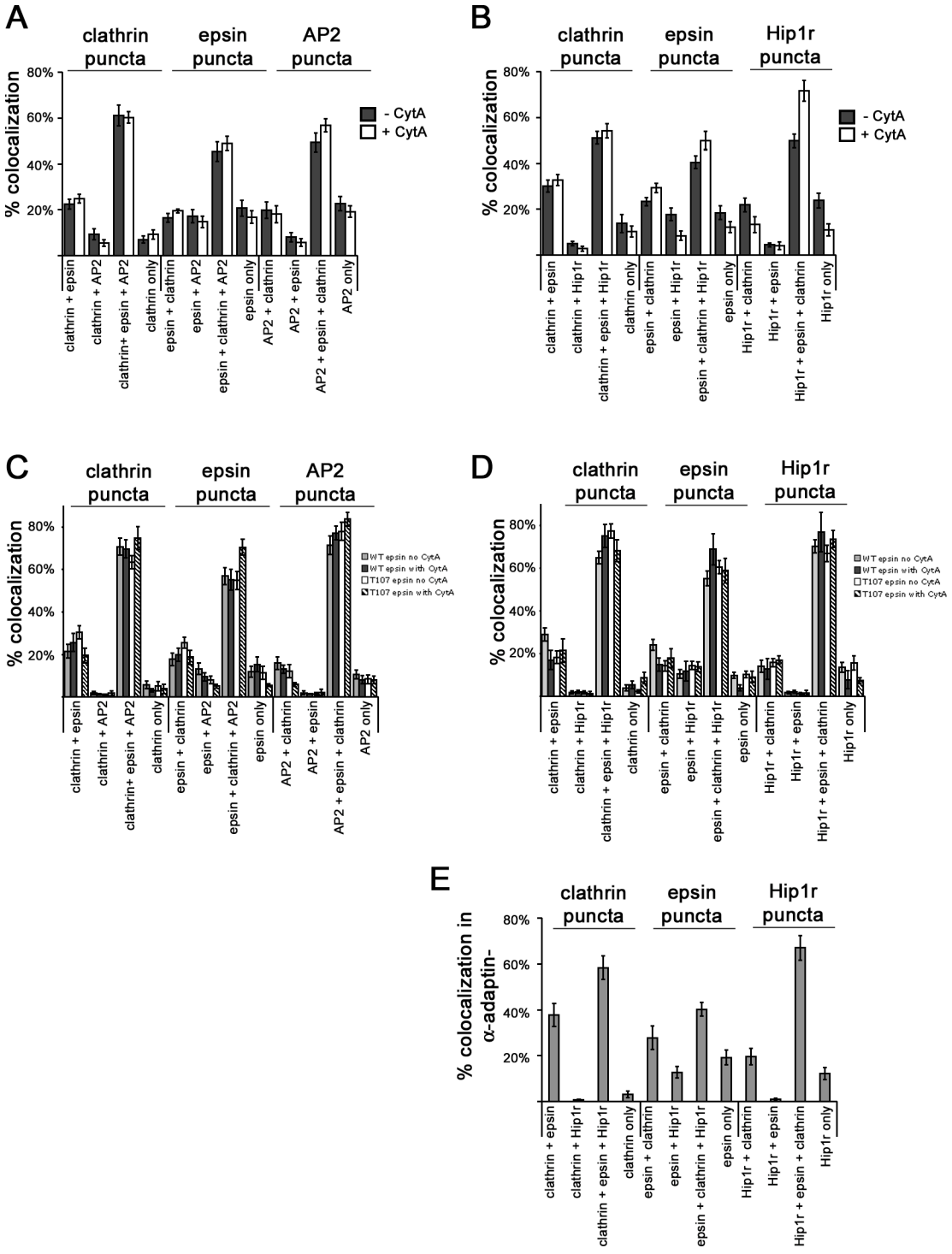

Figure D.17 Quantification of triple stain data

(Figure D.17, continued) Results of quantification of triple stain data using the macros from Appendix B. (A) Quantification of wild-type cells expressing CLC:RFP (**clathrin**) and epsinGFP (**epsin**) and immunostained with anti-α–adaptin antibodies (**AP2**). Cells were also treated with cytochalasin A (**+CytA**) before immunostaining. (B) Quantification of wild-type cells expressing CLC:RFP (**clathrin**) and epsinGFP (**epsin**) and immunostained with anti-Hip1r antibodies (**Hip1r**). Cells were also treated with cytochalasin A (**+CytA**) before immunostaining. (C) Quantification of epsin null cells expressing CLC:RFP (**clathrin**) and either epsin^{WT}GFP (**WT epsin**) or epsin^{T107A}GFP (**T107 epsin**). Cells were treated with cytochalasin A (**+CytA**) and immunostained with anti-α–adaptin antibodies (**AP2**). (D) Quantification of epsin null cells expressing CLC:RFP (**clathrin**) and either epsin^{WT}GFP (**WT epsin**) or epsin^{T107}AGFP (**T107 epsin**). Cells were treated with cytochalasin A (**+CytA**) and immunostained with anti-Hip1r antibodies (**Hip1r**). (E) Quantification of α–adaptin null cells expressing CLC:RFP (**clathrin**) and epsinGFP (**epsin**) immunostained with anti-Hip1r antibodies (**Hip1r**).

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Vita

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